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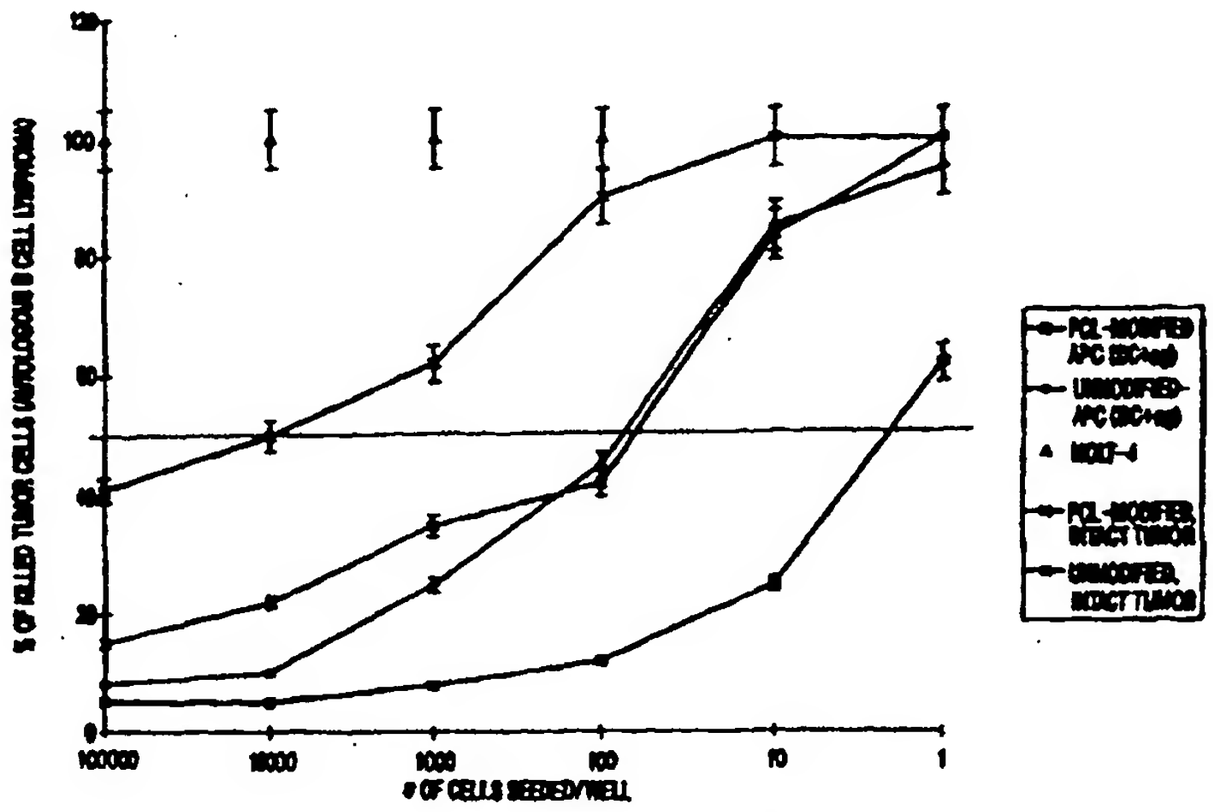
(57) Abstract

The invention describes a method of preincubating cells with dialdehydes, preferably a 2',3'-nucleoside or nucleotide dialdehyde as cross-linkers, prior to modifying the cell membrane components of the preincubated cells by exposure to hydrostatic pressure to produce potent immunogenic preparations. The pressure and crosslinker treatment of cells is termed PCL-modification. Preincubation PCL-modification allows the dialdehyde to serve as both a cross-linker of membrane components and a source of monoaldehyde groups on the cell surface, thus providing adjuvanting and costimulatory function to the cells. Also provided are immunogenic compositions comprising dialdehyde and antigen for augmenting and enhancing the immune response to a specific antigen. A reversible covalent chemical bond is formed between the dialdehyde component and the antigen component via one monoaldehyde of the dialdehyde. The other monoaldehyde can react with a biological response molecule or adjuvant for slow or prolonged release and enhancement of the immune response after immunization. Further provided are potent immunogens and immune response stimulators produced by PCL-treatment of antigen-presenting cells (APC) that have been pulsed or co-cultured with cancer, tumor or infected cell-associated antigens. The PCL-modified, antigen-pulsed APC can be used in immunogenic preparations and vaccines to increase immunogenicity and elicit vigorous cell-mediated responses and TH1 cytokine production. These cells can also be used with proliferated, antigen-specific T cells in immunogenic preparations and vaccines and in immunoadoptive therapies to treat and prevent cancers, tumors and infection. Biological response molecules, e.g., immunostimulatory cytokines, lymphokines and growth factors, can be coupled to the PCL-treated, antigen-pulsed compositions and APC to further enhance and augment the immune response.



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DIALDEHYDES AS IMMUNOSTIMULATORY ADJUVANTS AND CROSSLINKERS FOR PRODUCING IMMUNOGENIC PREPARATIONS AND GENERATING PRESSURE AND CROSSLINK TREATED CELLS FOR ENHANCING AND AUGMENTING THE IMMUNE RESPONSE AGAINST CANCER, TUMORS AND PATHOGENIC DISEASES.

FIELD OF THE INVENTION

The present invention is generally in the field of enhancement and augmentation of the immune response to tumor or cancer cells and to foreign antigens of pathogenic and/or infectious microorganisms. The present invention achieves enhancement and augmentation of the immune response by means of treating antigen presenting cells with crosslinker and hydrostatic pressure. The invention also relates to novel costimulatory adjuvants and molecules for use in prophylactic and therapeutic vaccine formulations and immunogenic preparations, to processes for producing potent vaccine and immunogenic preparations comprising pressure- and dialdehyde crosslinker-treated accessory immune cells, such as antigen presenting cells, and to the use of dialdehyde crosslinkers as novel costimulatory adjuvants for increasing and enhancing specific *in vivo* and *in vitro* immunogenicity against pathogenic diseases, tumors and cancers.

BACKGROUND OF THE INVENTION

Cancer and/or tumor cells frequently display on their external surfaces specific neo-antigens which are foreign to the immune system and immune cells of the host. However, for reasons that are not entirely clear, tumor or cancer cells often escape immune surveillance, and the immune system fails to develop an effective immune reaction against these cells. Attempts have been made to immunize cancer patients with preparations that will stimulate their immune systems to develop a reaction against the neo-antigens, with the hope that such an immune reaction will destroy the residing cancer or tumor.

The development of tumors and the transformation of normal cells to malignancy can result from a variety of different causes. Transforming events can occur spontaneously by random mutations, by gene rearrangement, or, they may be

induced by chemical, physical, viral, or microorganismal agents. Major classes of chemical carcinogens are known to include polycyclic aromatic hydrocarbons, such as are found in tar and soot, and aromatic amines, such as are found in certain dyes. Examples of physical carcinogens are X-rays and ionizing and ultraviolet radiation. In addition, both RNA and DNA viruses and viral oncogenes, are capable of transforming cells. When viral genes are introduced into cells, the infected cells are then triggered to express on the cell surface virus-associated antigens that can be recognized by the immune system. Moreover, the abnormal maintenance of certain viral oncogenes in a transcriptionally active state can result in transformation. In a similar manner, the infection of cells by bacteria or parasites or other microorganisms may lead to the expression of antigens at the cell surface and to recognition by immune cells.

The expression of at least some oncogenic, neo-antigenic, or "non-self" protein products, particularly those resulting from the mutation of a normal protein, should ideally render tumor, transformed, and infected cells sufficiently distinct from normal, unaffected cells so that the former can be detected by the immune system. However, as mentioned above, tumor and transformed cells, including cells infected with microorganisms that can affect the composition of the plasma membrane of the infected cells, often have the ability to avoid immunologic surveillance and detection.

In the case of tumors, the task of host immune surveillance is especially formidable because tumor cells and other abnormal cells have succeeded in escaping host surveillance by down-regulating the surface display of major histocomptability complexes (MHC) that are required for proper recognition by MHC-restricted CD4 and CD8 T lymphocytes. Other escape mechanisms, such as the down-regulation of costimulatory ligands on tumor cells or infected cells, may thwart the ability of responder T lymphocytes to be activated, thus resulting in an anergic response by the T lymphocytes. In addition, the stimulation of suppressor T lymphocytes can further attenuate the immune response. Thus, important problems and goals in tumor immunology and for the practitioner reside in determining what can be done to stimulate the cells of the immune system to

develop a reaction against tumor or non-self antigens with the end result of eradicating the tumor, cancer, infected or otherwise abnormal cells.

The relevant tumor antigens, or proteins, generally fall into two major categories: unique tumor specific antigens, which are found only on tumor cells and not on other host cells; and tumor associated antigens ("TAA") or determinants, which are found on tumor cells and also on some normal cells, but have qualitative and quantitative differences in expression on tumor and on normal cells. The best-studied unique tumor antigens are the neoantigens expressed on tumors induced in inbred mice by oncogenic viruses and chemical carcinogens. In contrast, spontaneous tumors, such as those induced by exposure to environmental carcinogens, have no predictable antigenic markers. However, it is pointed out that even if unique antigens are not found on human tumor cells, it may not be because such antigens do not exist, but because such antigens are difficult to detect, given the methods available in the art at the present time. Accordingly, needed in the art are ways to stimulate a strong and sustained immune response against tumor cells, which may have great potential for being highly immunogenic, but can successfully evade immune destruction.

The goal of therapies to treat and eradicate cancers and other types of transformed or infected cells is to provide efficient and safe vaccines and methods by which to increase the host's anti-tumor or foreign cell response against weakly immunogenic cell types, such as tumors and cells transformed by viruses or other pathogenic microorganisms.

Tumor and viral antigens elicit both humoral (antibody or B cell-mediated) and cell mediated immune responses *in vivo*, and virtually all of the effector components of the immune system have the potential to contribute to the eradication of tumor or virally infected cells. However, the T cell response is a most important host response for the control of growth of antigenic tumor cells, and transformed or infected cells, via cell-mediated immunity. The T cell response is effective for both the direct killing of tumor cells or infected (e.g., virus- or bacteria-infected) cells (by cytotoxic T cells) and the activation of other components of the immune system.

T cell immunity to tumors and infected cells involves the function of two T cell subsets: MHC class II-restricted T cells, which largely represent CD4 helper T cells (i.e., T_H), that mediate their effect by the secretion of lymphokines to activate other effector cells and to induce inflammatory responses; and MHC class I-restricted T cells, which represent CD8 cytotoxic T (T_C) cells, that also secrete lymphokines, but mediate their effect primarily by the direct lysis or killing of tumor cells. Because most tumor cells express class I, but not class II MHC molecules, the T_H cell subset cannot directly recognize these tumor cells. As a result, most T_H cell responses are dependent upon antigen-presenting cells or APC, such as macrophages, B lymphocytes and dendritic cells, to present the relevant tumor antigens in the context of class II MHC molecules for cell activation. Antigen presenting cells capture, process, and present most proteinaceous immunogens to the CD4 helper T cell subset. Following antigen-specific triggering, activated T_H cells secrete lymphokines that, in turn, activate T_C cells, macrophages, natural killer (NK) cells, and B cells; activated T_H cells also produce other lymphokines, such as lymphotoxin or tumor necrosis factor (TNF) which may also be directly lytic to tumor cells.

Recent findings have shown that two functional subsets of T_H cells exist. The first subset, the type 1 or T_H1 subset, appears to facilitate and then to reinforce primarily a cell-mediated immune response by cytotoxic T cells, i.e., T_C cells (see below); the second subset, the type 2 or T_H2 subset, appears to help B lymphocytes to mature and then to produce antibodies. The two T cell subsets are also distinguished by the types of cytokines that they produce. It has been demonstrated by *in vitro* studies that T_H1 cells release both interleukin-2 (IL-2) and interferon-γ (IFN-γ), while T_H2 cells release a combination of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and interleukin-10 (IL-10). Both IL-4 and IL-10 have been shown to shut down the cell-mediated immune response. Conversely, IFN-γ produced by T_H1 cells promotes cytotoxic T cell proliferation and inhibits antibody production. Thus, the various cytokines elaborated by the these two T cell subsets are cross-regulatory and can, when manipulated by a pathogen (e.g., virus, parasite or tumor cell) seriously suppress an individual's

effective immune response status to the particular pathogen.

Accordingly, the development of effective cancer or infected cell immunotherapy is believed to be primarily dependent on eliciting and sustaining a cell-mediated immune (CMI) response against the tumor or infection. This requires that CD4+ T lymphocytes be in "T helper type 1" (T_H1) state for enhancing the proliferation of cytotoxic CD8+ T cells, which is mediated through the secretion of specific T_H1-type cytokines (e.g., IFNγ, IL-2, TNF-β, IL-12, and the like) that suppress the T_H2 response. Conversely, CD4+ T lymphocytes in a T_H2-type mode secrete characteristic cytokines (e.g., IL-10, IL-4, and the like) which preferentially stimulate clonal expansion of B lymphocytes, resulting in antibody production, and inhibit CD8+ T cell proliferation. The balance between T_H1 and T_H2 cytokines can have a profound effect on the clinical outcome in a number of parasitic, chronic bacterial, mycobacterial, viral diseases, as well as in cancer. For example, in *Leishmaniasis major* infection in mice, animals which heal spontaneously exhibit an augmented cellular immune response with a dominant T_H1 profile against the pathogen. In contrast, animals which exhibit a high antibody titer coupled with expansion of T_H2 cells develop chronic disease (F.P. Heinzel et al., 1991, *J. Exp. Med.*, 169:59-72. An analogous situation is seen in human leprosy where patients with persistent infection exhibit predominant humoral response and a T_H2 type cytokine pattern, while patients who heal display a strong cellular response associated with T_H1 cytokine pattern (P. Salgame et al., 1991, *Science*, 254:279-282. Recent reports suggest that a number of human tumors, such as melanoma, glioblastoma multiforme and bronchogenic carcinoma principally secrete IL-10 with only trace amounts of T_H1 cytokines. Matsuda et al. (1991, *J. Exp. Med.*, 180:2371-2376) have shown that IL-10 can protect tumor cells from tumor- and allo-specific cytotoxic T cells, as well as down-regulate HLA class I expression, suggesting that this might be a general mode by which tumors modulate the immune response to their advantage.

With particular regard to HIV infection, disease, and pathogenesis, it has been observed that as patients progress toward AIDS disease, there is a change in the patient's immune response to the virus, such that they move away from a

T_H1-type response (i.e., predominantly cell-mediated) to a less effective T_H2-type response (i.e., primarily antibody production). Thus, a "T_H1 to T_H2 switch" might be involved in the progression toward AIDS (C. Ezzell, 1993, *The J. of NIH Research*, 5:59-64).

In contrast to the action of T_H cells and subsets thereof, class I-restricted T_C cells are capable of directly recognizing and killing tumor target cells by disrupting the target cell membrane and nucleus (Bjorkman, P. et al., 1990. "Structure, function, and diversity of class I major histocompatibility complex molecules", *Ann. Rev. Biochem.*, 59:253). Only a minor fraction of class I-restricted T cells is capable of providing helper functions; thus, effective T_C cell responses are generally dependent upon class II-restricted T_H cell responses to provide the necessary helper factors to activate and promote the proliferation of T_C cells. The T cell receptor of an antigen-specific T_C cell clone recognizes class I MHC-peptide complexes which, after intracellular processing of viral or tumor antigens appear, at the surface of virally infected or transformed cells, for example. Cytotoxic T cells become activated to eradicate foreign cells by releasing toxins or inducing the target cell to commit suicide, perhaps by physical contact with the foreign target cell. The activated T_C cells proliferate and give rise to additional T_C cells having the same antigen specificity. As mentioned above, the role of antigen-presenting cells (APC) is to offer antigenic peptides complexed with MHC molecules to the available repertoire of T cells. Thus, MHC molecules are obligatory components of the immunogenic complex recognized by T cells and play a central role in the immune response to foreign and tumor antigens. The ability of T cells to recognize specific features of MHC proteins is crucial for the immune system to function properly and to discriminate "self" from "non-self".

Accordingly, before they can help other lymphocytes respond to foreign antigens, T_H cells themselves first must be activated. Activation occurs when a T_H cell recognizes a foreign antigen bound to a class II MHC glycoprotein on the surface of an APC. APC are found in most tissues. They are derived from bone marrow and comprise a heterogeneous set of cells, such as dendritic cells in lymphoid organs, Langerhans cells in skin, and certain types of macrophages.

Populations of APC can also be isolated and expanded for use in stimulating an immune response (with particular regard to dendritic cells, see, for example, WO 93/20185 to R.M. Steinman et al. and WO 91/13632). Together with B lymphocytes, which can also present antigen to T_H cells, and thymus epithelial cells, such specialized APC are the main cell types that normally express class II MHC molecules. Moreover, cells such as fibroblasts, which do not make class II MHC molecules and cannot present foreign antigens to T_H cells, can be converted to effective APC if they are transfected with a gene encoding a class II MHC molecule. In addition, other cells that normally do not express class II MHC glycoproteins, such as endothelial cells, can be induced to express these proteins following the production of interleukins, such as gamma interferon by activated T_H cells. The sequence of events leading to the presentation of antigen to T cells at the cell surface of APC is known as antigen processing (see, Molecular Biology of the Cell, 2nd Ed., Eds. B. Alberts et al., 1989, pages 1045-1048).

Tumor cells, cancer cells, and otherwise transformed cells may lose their regulated proliferative state due to the acquisition of means by which to cause disease, grow uncontrollably, and invade distant tissue. Such cells may also display unique or characteristic tumor associated antigens on the cell surface and the processed antigens may be complexed with class I or class II MHC structures for presentation to the effector cells of the immune system. Similarly, cells transformed by viruses, bacteria, parasites, and the like, may also present processed antigens, peptides, proteins, or structures of the foreign pathogen, microorganism, or other infectious agent (e.g., prions), complexed with class I or class II MHC molecules for presentation to the effector cells of the immune system. In an optimal situation, such foreign antigens are presented by or complexed or associated with MHC class I structures on the surfaces of infected or transformed cells for recognition and efficient killing by cytotoxic T cells.

A potential solution to the problem of overcoming the ability of cancer, infected or transformed cells to avoid host immune surveillance is to manipulate and amplify the immune system and its cellular components in order to promote the eradication of the abnormal cells. This solution is also conducive for

eliminating cells infected or transformed by other exogenous agents, e.g., viruses and other microorganisms such as parasites, protozoa, algae, yeast, fungi, and bacteria. Provoking an effective immune response against malignant cells requires the elimination of control mechanisms utilized by cancer cells to conceal their identity antigens. For T-cell recognition to occur, cancer antigens must expose and express themselves above a critical threshold in conjunction with MHC molecules.

In the field of immunotherapeutic vaccines and immunogenic preparations, there is a need for novel and potent vaccines, immunogenic preparations, and methods to modify or modulate the host's response to abnormal cells to induce an increased response to the tumor or tumor load, to infected cells, and to cells displaying non-self antigens. There is also a need in the art for successful immunotherapeutics and immunotherapy techniques which result in reduction of tumor mass, and, ultimately, in the complete eradication of tumor or infected cells.

Because of the multiplicity of tumor antigens, a multivalent vaccine is needed which comprises the widest repertoire of tumor antigens and which specifically augments the cellular immune response against a weakly immunogenic tumor. Such is afforded by the present invention.

The efficacy and potency of vaccines and immunogenic preparations for use in reducing or eradicating cancers and infected cells may be increased by the use of adjuvants, which are agents, e.g., compounds, chemicals, proteins, molecules, lipids, and the like, which augment specific immune responses. Adjuvants can serve as immunostimulatory or immunopotentiating agents, alone or in combination with other compounds, to improve or increase the host's immune response and resistance to a tumor or invading pathogenic microorganism or virus. Immunopotentiating or immunostimulatory agents are capable of stimulating, enhancing, or increasing an immune response to foreign antigen. It is also noted that such agents may also suppress immune function or responsiveness, if the timing, mode, and route of administration are conducive to such immunosuppression. Adjuvants as immunopotentiators or immunostimulators can increase immune resistance to antigen in a vaccine preparation and to tumors and

infected cells in a vaccine preparation employing tumor or infected cells.

Until the present invention, the commonly used compounds or materials having adjuvant activity included, for example: alum and similar aluminum gels, which are approved for use in humans; Freund's incomplete adjuvant; Freund's complete adjuvant; Quil A or saponin; muramyl dipeptide; iscoms or lipid micelles; non-metabolized synthetic molecules which are capable of binding proteins to cell surfaces; and nonionic block copolymer surfactants. The use of alum as adjuvant poses problems because it does not stimulate cell mediated immunity; it provides low yields of effective vaccines when small peptides are used as vaccines due to inadequate adsorption; and it may cause degradation of antigens via the induction of protease activity. The materials other than alum are frequently not optimal for use in mammals, especially humans, due to their ability to cause chronic reactions and toxicity in the hosts.

Small Schiff-base forming molecules, i.e., substituted benzaldehyde tucaresol, and monoaldehydes have been reported to provide costimulatory signals to cells by donating carbonyl groups through a mechanism that activates clofilium-sensitive potassium and sodium transport (J. Rhodes et al., 1995, *Nature*, 377:71-75; X-M. Gao et al, 1990, *J. Immunol.*, 144:2883-2890). A significant drawback to the use of tucaresol and other such molecules as costimulatory agents or adjuvants, as demonstrated by Rhodes et al., is that their functional usefulness *in vivo* is over an extremely narrow concentration range. Such a narrow range makes these molecules difficult to control and unrealistic for physiological use. It has also been shown that the use of other aldehydes, such as dialdehydes, caused inhibition of antigen-specific T cell activation (J. Rhodes et al., 1992, *Immunology*, 75:626-631).

NAGO, another adjuvant system, has been used to co-treat cells by first treating cells with neuraminidase (NA), which oxidizes cell surface galactose residues on membrane glycoproteins, followed by treating with galactose oxidase (GO), which converts the galactose to an aldehydic structure causing a biochemical derivatization of cell surface aldehydes. NAGO treatment has been shown to result in lymphocyte blastogenesis and transformation *in vitro* (A. Novogrodsky and E.

Katchalski, 1973, *Proc. Natl. Acad. Sci. USA*, 70:1824-1827) and enhancement of immunogenicity in vivo (J. Rhodes et al., 1995, *Ann. NY Acad. Sci.*, 754:169-186; and WO 92/03164). In addition, the *in vivo* use of NAGO may be toxic and may not be well tolerated in mammalian hosts. In addition, the use of this technique is likely to suffer from lack of specificity *in vivo*, especially in the narrow and high-level dose range required for efficacy and activity and after repeated administration in mammalian hosts.

Thus, it is apparent that novel anti-tumor cell and anti-infected cell vaccines and immunogenic preparations, along with new, safe, and effective adjuvanting compounds and compositions, are needed in the art for use in dose ranges that are both non-toxic and useful for enhancing immune responsiveness to a variety of antigens associated with cancer and pathogenic disease-causing organisms after immunization.

SUMMARY OF THE INVENTION

In one of its aspects, the present invention provides the use of dialdehydes as stimulators, potentiators, and adjuvants to enhance and increase the immune response to antigens, particularly tumor antigens, antigens derived from viruses, bacteria, yeast, fungi and other pathogenic disease-causing microorganisms. The dialdehydes, when reacted with cells or antigen, modify the cell membrane components or antigen by forming transient covalent chemical reactions such that one aldehyde group of the dialdehyde reacts as a monoaldehyde with a cell membrane component or antigen, and the other pendant monoaldehyde group is free to react with other cell membrane ligands or non-membrane bound ligands, such as protein or carbohydrate. Dialdehydes function as adjuvanting molecules, immunostimulatory molecules and immunopotentiating molecules in the methods and compositions of the invention to achieve potent immunogens and vaccines comprising dialdehydes. The dialdehydes also may provide the costimulatory signals that are required for activation and response by T cells; in this way the dialdehydes of the invention may replace the need for costimulatory molecular interactions traditionally supplied by antigen-presenting cells.

It is an object of the invention to provide a improvement and

modification of the pressure and crosslinker treatment (PCL) of cells, e.g., tumor cells, cells infected or transformed with bacteria and pathogenic disease-causing viruses and microorganisms, and bacterial cells, and the like, by preincubating the cells in dialdehyde prior to subjecting the cells to the PCL modification process. In the improvement method, the dialdehyde functions as both a crosslinker to react with and crosslink cell membrane components within the cell surface, and as an adjuvant molecule wherein its two monoaldehydic groups are able to form reversible chemical covalent linkages with cell membrane components and/or with non-membrane-bound components. The amount of monoaldehyde produced and available for further interaction with other components can be quantified after incubation of cells with dialdehyde.

It is another object of the invention to provide novel and potent compositions and formulations comprising a dialdehyde component and a component comprising cells containing antigen(s) on their surfaces for use as immunogens and vaccines to enhance and augment the immune response to the specific cellular antigen(s) of the composition. The cells component of the composition is preferably PCL modified.

It is another object of the invention to provide novel and potent compositions and formulations comprising a dialdehyde component and an antigen component for use as immunogens and vaccines to enhance and augment the immune response to the specific antigen of the composition. The antigen component of the composition is preferably not bound to a cell.

It is yet another object of the invention to provide vaccine and immunogenic formulations comprising dialdehyde and antigen, wherein one aldehyde group of the dialdehyde is linked to antigen and the other aldehyde group is linked to another adjuvant, immunopotentiating or immunostimulating agent, such as a cytokine, a lymphokine, or a chemokine.

It is a further object of the invention to provide a composition comprising dialdehyde coupled or linked to any immunogen, either cellular or noncellular, for example, an antigen such as protein, peptide, or carbohydrate, for use as therapeutic and prophylactic vaccines to enhance and augment the immune

response to the immunogen.

It is yet another object of the invention to provide methods for treating a mammal, including humans, by administering the dialdehyde and antigen-containing compositions and formulations to patients afflicted with a tumor or a pathogenic disease or infection.

It is yet another object of the invention to utilize PCL treatment to create potent vaccines involving antigen-presenting cells (also called accessory cells) for the treatment and prevention of cancers, tumors and infectious diseases caused by pathogenic microorganisms. Such vaccines are also useful for the *ex vivo* generation of specific cytotoxic T lymphocytes (CTLs), which may be particularly employed in the treatment of cancers such as B-cell lymphomas, and in treating and preventing the growth of tumors and infected cells. In accordance with the invention, PCL treatment is performed on antigen-presenting cells or accessory cells to augment the capacity of these cells to elicit a vigorous immune response, ultimately activating T cells to target antigens, such as those associated with tumors, cancers and infectious microorganisms to reduce cancer, tumor or infected cell load and/or to eradicate the system of cancer, tumor and infected cells.

Further objects and advantages afforded by the invention will be apparent from the detailed description hereinbelow.

BRIEF DESCRIPTION OF THE DRAWINGS

In describing the invention, reference will at times be made to the accompanying drawings in which:

Fig. 1 shows a functional assay of 2′, 3′-adenosine dialdehyde (AdA) determined by fluorescence and presented for three active and purified preparations (batches) of the crosslinker (see Example 1C).

Fig. 2 shows an evaluation of the binding kinetics of AdA to B16 melanoma cells as a function of pH (see Example 1D)

Fig. 3 shows the determination of the percent occupancy of available cell surface binding sites for AdA on B16 melanoma cell surfaces after reacting cells with this dialdehyde as a function of time (see Example 1E).

Fig. 4 sh ws the results of analyses to determine the number of AmA molecules bound per cell and the effect of chemical reducing agents on lowering the amount of membrane-bound AdA (see Example 4).

Fig. 5 shows the results of IVS analyses to determine the immunological effects of preincubating cells in dialdehyde and then PCL-treating the preincubated cells by exposure to AdA crosslinker and hydrostatic pressure.

Fig. 6 demonstrates anti-tumor effectiveness in the presence of cell membrane bound adenosine monoaldehyde in vivo.

Fig. 7 demonstrates the effects of dialdehydes as adjuvants/immunopotentiators of the cellular response to immunoproliferating agents.

Fig. 8 shows the results of cytotoxic T cell assays using PCL-modified lymphoma cells and PCL-modified dendritic cells, in which cytotoxic T cells are stimulated to react against autologous human lymphoma cells. Increased precursor frequency of anti-tumor CTL is seen following stimulation with PCL-modified intact tumor cells or loaded (+ antigen) dendritic antigen presenting cells (APC).

Fig. 9 shows the augmentation of the proliferative response of primary T cell lines to intact, irradiated tumor cells (EBV-associated B cell lymphoma) following PCL-modification of the B lymphoma cells (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; open triangles: K562.

Fig. 10 shows the secretion of higher levels of γIFN by primary T cell lines following stimulation with PCL-modified B cell lymphomas (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; closed triangles: K562.

Fig. 11 shows the secretion of lower levels of IL-10 by primary T cell lines following stimulation with PCL-modified B cell lymphomas (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; closed triangles: K562.

Fig. 12 shows the augmentation of the proliferative response of

primary T cell lines to antigen presenting cells, i.e., dendritic cells (DC), pulsed with allogeneic B cell lymphoma extract following PCL-modification of the antigen presenting cells (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; closed triangles: K562.

Fig. 13 shows the increased presentation of tumor-specific antigens to T cells, demonstrated by enhanced secretion of γIFN, following PCL-modification of APC (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; closed triangles: K562.

Fig. 14 shows that primary T cell lines secrete decreased levels of IL-10 upon stimulation with dendritic cells pulsed with B cell lymphoma extract and modified by PCL (Example 17). On graphs, closed squares: autologous B cell lymphoma; closed diamonds: HLA-A matched B cell lymphoma; closed triangles: K562.

Fig. 15 shows that PCL modification of intact tumor cells or dendritic cells (DC)-pulsed with tumor antigen elicits the proliferation of both CD4+ and CD8+ cells. Black bars: intact BLCL; white bars: DC-pulsed with tumor cells.

Figs. 16A-16D present profiles of the levels of chemokines and lymphokines produced by T cells during *in vitro* stimulation assays with BLCL that have been PCL modified or unmodified as controls. Fig. 16A: IFN-gamma profile; Fig. 16B: MIP-1-beta profile; Fig. 16C: MIP-1-alpha profile; Fig. 16D: RANTES profile.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to an improved method of treating tumor or infected cells with hydrostatic pressure and a dialdehyde chemical crosslinker to result in immunogens having increased and heightened immunogenicity for stimulating a specific immune response in the host. The immunogens may be whole tumor cells or infected cells, or they may be pathogenic disease-causing microorganisms or cells treated as described; they may also be

plasma membranes derived from these cells; or specific immunogenic proteins obtained from the tumor, infected or pathogenic cells or cell membranes thereof. Infected cells are those that are infected and/or transformed with viruses or other pathogenic microorganisms or agents as described herein. The treatment of cells with pressure and crosslinker is termed PCL modification, PCL treatment, or PCL technology as used herein. The invention further relates to the use of dialdehyde both as the aforementioned crosslinker and as an adjuvanting, immunostimulatory or immunopotentiating molecule, whether on the surfaces of tumor or infected cells or in compositions with antigen useful as immunogens and vaccines.

The invention further relates to therapeutic and prophylactic vaccines and immunogens comprising PCL-modified and unmodified antigen-presenting cells (APC) and PCL-treated tumor, cancer, or infected cells, and/or proteins and peptides derived therefrom. In accordance with the invention, APC may include, but are not limited to, dendritic cells, fibrocytes, astrocytes, macrophages, B cells, microglial cells and fibroblasts. Fibrocytes are a preferred APC type and are characterized by their rapid entry from blood into subcutaneously implanted wound chambers and by their presence in connective tissue scars, as described by R. Bucala et al., 1994, *Mol. Med.*, 1:71-81.

The APC can be activated or unactivated, i.e., pulsed or not pulsed with antigen. In addition, the APC may be genetically engineered to present antigen in the context of MHC glycoproteins, and as such, may be termed "synthetic" APC. Immortalized APC are also suitable for use in accordance with the invention. APC can be immortalized via infection with viruses, e.g., Epstein Barr Virus (EBV), human papilloma virus (HPV) and the like); transfection of cells with plasmids containing oncogenes, e.g., SV40 and the like, and other sequences or elements which allow for appropriate transcription, translation, expression and processing of encoded polypeptides in host cells, such as promoters, enhancers, terminators, poly A sequence, and the like; or via mutagenesis, e.g., using chemical mutagens alone or in combination with ultraviolet radiation. Preferred are PCL-modified populations of APC that are pulsed with antigen. More preferred are dendritic cells, which may be employed as an essentially

homogeneous population of expanded cells, or as a more heterogeneous population isolated from the appropriate tissue source.

PCL technology has been described in U.S. Patent No. 5,582,831, in application U.S. Serial No. 08/392,553, filed February 17, 1995 and in application U.S. Serial No. 08/481,857, filed June 7, 1995, the contents of which are incorporated herein by reference in their entirety. This technology provides a unique and innovative approach for the creation of whole cell vaccines or immunogens comprising PCL-modified tumor cells or infected cells, which are safe and simple to manufacture.

As used herein, vaccines, vaccine formulations, compositions and preparations may be either prophylactic and therapeutic. Immunogenic formulations, compositions and preparations may be considered to be vaccines by those having skill in the art. PCL vaccines or immunogens are those comprising cells that have been modified by crosslinking and hydrostatic pressure treatment.

The improvement to PCL treatment of cells yields potent immunogens for use as anti-cancer and anti-infected cell vaccines both with and without exogenous adjuvants. The application of pressure to cells exerts powerful biological and physical effects on cellular components. At the biological level, pressure results in a reversible dissociation of the cytoskeleton (B. Bourns et al., 1988, *Cell Motil. Cytoskel.*, 10:380-390), as well as the subsequent transient induction of protein synthesis, in particular, the synthesis of stress-related proteins called pressure-induced proteins (PIPs) such as transporter or chaperone proteins, which participate in the recovery from pressure-induced stress. At the physical level, intense pressure can increase the density of cell membranes with rigidifying effects (T.J. Welsh et al., 1993, *J. Bact.*, 175:7170-7177). The immunological consequences of pressure on tumor cells have also been described (M. Shinitzky, 1984, *Biochim. Biophys. Acta*, 738:251-261; V. Ramakrishna and M. Shinitzky, 1991, *Cancer Immunol. Immunother.*, 33:1-8; V. Ramakrishna et al., 1993, *Cancer Immunol. Immunother.*, 36:293-299; and A. Eisenthal et al., 1993, *Cancer Immunol. Immunother.*, 36:300-306).

The molecular mechanisms by which pressure-modified tumor cells

become more immunogenic are not fully understood. As mentioned above, recent studies suggest that pressure can cause the induction of the transient appearance of PIPs. In addition, in two separate cell systems (i.e., *E. coli* and osteosarcoma) the application of a brief intense hydrostatic pressure to these cells resulted in the specific synthesis of transporter proteins, known as heat shock proteins (HSP) (C.L. Haskin et al., 1993, *Biochem. Cell. Biol.*, 71:361-371; T.J. Welsh et al., *J. Bacteriol.*, 175:7170-7177). HSP are essential for the transport of MHC/antigen complexes from intracellular compartments to the cell surface and have been shown to be down-regulated in several tumor cells (D. Ang et al., 1991, *J. Biol. Chem.*, 266:24233-24236; P.K. Srivastava, 1993, *Adv. Cancer Res.*, 62:153-177). It has also been recently shown that a number of HSP derived from tumor cells are potent immunogens by virtue of the tumor antigens which are adsorbed onto them (P.K. Srivastava et al., 1994, *Immunogenetics*, 39:93-98; H. Udono et al., 1994, *Proc. Natl. Acad. Sci. USA*, 92:3077-3081). Several of these heat shock proteins were found by the inventors to be induced during PCL of tumor cells (B16).

That active biological processes take place in the course of PCL modification of cells was recently demonstrated by the present inventors in studies in which pre-treatment of tumor cells with a protein synthesis inhibitor (cycloheximide) was found to block approximately half of the MHC presentation on the cell surface. PIPs other than HSP may also be expressed during PCL treatment of cells; indeed, data of the inventors suggest that other immunologically relevant molecules such as β_2M and adhesion molecules such as ICAM-1 are induced or exposed at the cell surface after PCL modification. In addition, since the murine B16 melanoma is known to lack the costimulatory ligand B7-1, and since PCL-modified B16 cells become fully immunogenic, preincubation of cells in dialdehyde, PCL treatment, and the dialdehyde crosslinker and pressure components thereof, may cause the expression of this ligand and/or provide other costimulatory signals to cells, as is described further herein.

Without wishing to be bound by any particular theory and in view of the foregoing, the elevated immunogenicity of tumor cells following subjection to PCL may result from (i) endogenous peptides forced into the class I and class II

intracellular compartments where they are assembled with MHC molecules, routed, and transported through appropriate pathways to be expressed in an extended concentration on the cell membrane; (ii) removal of repressors utilized to inhibit the expression of cancer identity antigens and consequent alleviation of such inhibitory control; (iii) induced changes in the fluidity of the cell membrane, giving rise to an unusual display of self proteins; (iv) active induction of a wide range of proteins such as heat shock proteins, tumor specific antigens and MHC, with documented contribution to T-cell recognition of transformed or infected cells; and (v) co-stimulatory function of the free aldehyde generated by the dialdehyde crosslinker (AdA) and activation of T-cells through a Schiff base reaction, resulting in amplification of T-cell activation.

In one embodiment of the present invention, tumor cells are modified by an improvement to the basic PCL method to produce an immunogen derived from modified tumor cells and capable of inducing an anti-tumor immune response, wherein the modified tumor cells have been prepared by first preincubating the tumor cells with dialdehyde crosslinking agent, and then exposing the preincubated cells to the dialdehyde crosslinking agent and to hydrostatic pressure at a concentration and for a time sufficient to cause a modification of proteins in the cells' plasma membranes. Preferred is exposure of the preincubated cells to crosslinking agent and to hydrostatic pressure at the same time, following the preincubation. The preincubation was found to insure that the resulting immunogenicity of the PCL-modified tumor cells in the immunogenic vaccine preparation was fully controlled and afforded a consistent protective response *in vivo* and a consistent proliferation or stimulation response in *in vitro* stimulation assays.

In another embodiment, infected or otherwise transformed cells are modified by the improved PCL method to produce an immunogen derived from the preincubated and PCL-modified infected or transformed cells, which is capable of inducing an immune response against the infecting or pathogenic disease-causing agent, wherein the modified cells have been prepared by first preincubating the infected cells with dialdehyde crosslinking agent, and then exposing the

preincubated cells to crosslinking agent and to pressure at a concentration and for a time sufficient to cause a modification of proteins in the cells' plasma membranes. In a preferred embodiment, after preincubation, cells are exposed to crosslinking agent and to hydrostatic pressure at the same time.

An alternative embodiment encompasses direct PCL treatment, with and without dialdehyde preincubation, of bacterial cells, as well as other pathogenic disease causing cells, to augment relevant antigen expression and the use of the PCL-modified whole cell bacteria, and the like, in PCL vaccines. PCL treatment of whole cell pathogenic bacteria and other cellular pathogenic organisms can result in the expression of greater levels of the components that correlate with increased virulence indices.

In another embodiment, APC are PCL-treated in accordance with the improved invention and the PCL-treated APC are used in vaccine and immunogenic preparations. PCL-treated APC can also be used in IVS cultures to proliferate antigen-specific CTLs *in vitro* for use in immunoadoptive therapy. In addition, the PCL-treated APC can be employed in combination with the proliferated CTLs and used as an active component in immunoadoptive therapy. Non-limiting examples of APC suitable for use include dendritic cells, macrophages, astrocytes, fibroblasts, fibrocytes, Langerhans cells and endothelial cells. It is also envisioned that the aforementioned types of APC can be immortalized or genetically engineered to express exogenous genes to allow, enhance, or improve antigen presentation in the engineered cells.

In general, antigen-specific T cells may be generated using professional APC (e.g., DCs, fibrocytes, macrophages and the like) in the following manner. The antigen(s) toward which the T cells are specifically activated can include those derived from tumors, cancers, viruses, parasites, bacteria, and the like. T cells obtained from blood, lymph nodes, tumors or lesions are incubated with professional APC that have been previously pulsed with antigen or antigen extract, e.g., cell extract, subcellular fractions, purified proteins or peptides. The T cells are can be purified by conventional methods, such as FACS, magnetic beads, positive or negative selection and the like), or they can be

stimulated as heterogeneous cell suspensions. Professional APC can also be added to co-cultures of T cells with intact stimulator cells as "third party" cells. The stimulator cells may be tumor or cancer cells, virus-infected cells, or bacteria- or parasite-infected cells, for example.

Cultures of T cells and APC (e.g., DC) pulsed with antigen or T cells + stimulator cells + APC (DC) are incubated for about 10 to 14 days at 37°C and 5% CO_2. During this period, a subpopulation of T cells with the appropriate T cell receptor (TCR) specificity are activated and expanded. These expanded T cells can be infused into patients according to conventional adoptive therapy regimes. The T cell culture can be kept for up to 21 days before requiring additional antigenic challenge. T cell cultures can be rechallenged with APC (DC) and antigen at intervals of 14 to 21 days and for as many times as is required by the ongoing therapy.

This aspect of the invention also encompasses mixtures of several types of APC. For example, DC are extremely potent APC and must be used at the correct ratios, i.e., T cells: APC : antigen, to avoid the possibility of apoptosis upon additional restimulation or challenge with antigen. The correct ratios are empirically determined as known to those in the art and may change according to culture conditions, state of the cells and type of antigen. Therefore, in some cases, the effects of potent APC are attenuated by dilution of their numbers by the addition of less efficacious APC, such as B cells, fibrocytes, or macrophages, (also called filler cells, which are irradiated (3000 rad) leukocytes/lymphocytes) to the more potent APC cultures. The nature of the antigen may also dictate the use of a mixture of APC. If the antigen is soluble, fibrocytes and B cells may be sufficient for antigen processing and presentation. Accordingly, the addition of DC to cultures of B cells or fibrocytes increases the signals due to the high density of co-stimulatory molecules or carries out antigen processing of particular proteins found in an antigen preparation. The preparation of non-PCL-treated B cells, macrophages and DC for use as APC is known to those having skill in the art.

The APC may be co-cultured with intact autologous cancer or tumor cells, or with intact autologous cells containing intracellular pathogens (e.g., cells

infected with viruses, bacteria, parasites, yeast, algae, fungi, and the like, which express antigens associated with the pathogenic organism). The autologous cancer, tumor or infected cells are preferably PCL-modified or PCL-modified and coupled to biological response modifiers (BRMs) as described herein. Further, the APC may be co-cultured with intact allogeneic cancer or tumor cells, or with intact allogeneic cells containing intracellular pathogens, as described above. The allogeneic cancer, tumor or infected cells are preferably PCL-modified or PCL-modified and coupled to biological response modifiers as described herein. Before or after co-culture, the APC can be unmodified, PCL-modified, or PCL-modified and coupled to BRMs. Preferred are APC that have been PCL-modified or PCL-modified and coupled with BRMs in accordance with the improved methods of the invention. In general, the cancer or tumor cells may be derived from an intact cancer or tumor, an excised cancer or tumor, or may be immortalized or transformed cell lines. APC can be co-cultured with disrupted cells, cell membranes, cell fragments and subcellular fractions prepared by methods known to those having skill in the art.

In addition, APC can be pulsed with a variety of immunogenic agents to allow the endogenous uptake of these agents, processing and antigen-presentation on the surface of the APC. Examples of the types of agents with which APC can be pulsed include, but are not limited to, autologous or allogeneic whole cell lysates or subcellular fractions thereof; defined peptides or proteins, combinations of different peptides, combinations of different proteins; antigens harvested from cancer or tumor cells wherein the cells are autologous, allogeneic or grown as cell lines; antigens harvested from cells infected with viruses and other pathogenic microorganisms; synthetic antigens, including peptides and polypeptides; and isolated RNA. Some nonlimiting examples of peptides and/or proteins that can be used for pulsing include melanoma tumor-specific antigens, such as MART-1, MAGE-1 and Gp-100; breast and ovarian cancer tumor specific antigens, such as HER-2neu; EBV-associated antigens, such as EBNA1-3 and LMP-1 or 2; HPV-cervical cancer-associated antigens, such as E5, E6, E7, L1 and L2; Hepatitis B and C-derived antigens; and HIV-associated antigens. Before or

after pulsing, the APC can be unmodified, PCL-modified, or PCL-modified and coupled to BRMs. Preferred are APC that have been PCL-modified or PCL-modified and coupled with BRMs in accordance with the improved methods of the invention.

APC, e.g., DC, are pulsed with antigen by incubation in the presence of commercially-available liposomes or naked proteins. The time of incubation is dependent upon the particular antigen. Peptides bind to surface MHC within minutes. Antigens which require processing, i.e., peptides containing about 20 or more amino acids; proteins; membrane fragments or cell fragments, are usually incubated with APC for about 5 to 16 hours. The rate and final dose of antigen uptake are dependent upon a given APC. The antigen is usually present in excess, for example, about 10 to 100 μg/ml of protein or peptide, or about 1-0.05 equivalent tumor cell extract per APC. Conventional methods for purification of APC are used and known among those having skill in the art. PCL treatment of APC is preferably carried out after pulsing APC with particular antigen or polypeptide. After PCL treatment, APC frequently lose their capability to process antigen, e.g., proteins, cell fragments and the like, as described above. Therefore, the presentation of short peptides is not compromised. If infected cells or tumor cells are the antigen source, such cells can be PCL-treated, disrupted and used to pulse APC. These APC can then be PCL-modified following the completion of the incubation period with antigen.

The co-cultured or antigen-pulsed APC can also be used in immunogenic preparations comprising T lymphocytes which have been proliferated with cells bearing antigens associated with particular cancers, tumors or infectious organisms. The method generally involves obtaining a tissue source of APC (e.g., adult, fetal or cord blood, bone marrow, spleen or afferent lymph); enriching, expanding and/or proliferating the APC by methods known in the art; exposing the APC via co-culture or antigen pulsing to cancer, tumor or infected cell-associated antigens to achieve antigen presentation and activation by the APC; incubating the antigen activated APC in dialdehyde crosslinker, particularly, 2′, 3′ nucleotide or nucleoside dialdehyde crosslinker from about 15 to 30 minutes, treating the

crosslinker treated APC with hydrostatic pressure of about 500 to 1400 atmospheres, preferably 1000 to 1200 atm and washing the PCL-modified APC. The PCL-treatment of antigen presenting cells can occur prior to or after exposure to cancer, tumor or infected cell-associated antigens; PCL-treatment after co-culture or antigen pulsing is preferred.

T cells from a human peripheral blood mononuclear cell source are expanded and proliferated by IVS assay in which the stimulator cancer, tumor or infected cell antigens are displayed on intact cells or plasma membranes thereof. The stimulator cells are preferably PCL-treated in accordance with the invention prior to their use as stimulators in the IVS assay. In accordance with the invention, following PCL modification, the intact cancer or tumor cells, and/or antigen-pulsed APC demonstrate an increased immunogenicity and an immense capacity to elicit a vigorous anti-cancer, tumor or infected cell immune response. More specifically, cytotoxic T cells triggered by the above-PCL-modified cells secrete elevated levels of cytokines and lymphokines of the TH1 type to produce an active and specific cell-mediated response which targets and removes cancer, tumor or infected cells.

Exemplification of this embodiment of the invention was carried out using B cell lymphoma as a model cancer system and dendritic cells as model APC. Dendritic cells (DC) pulsed with cell extract prepared from a pool of various allogeneic EBV-associated B-cell lymphoma were used as a cellular anti-cancer vaccine to generate tumor-specific T-cell lines. DC-pulsed with tumor antigens ("loaded APC") derived from the cell extract were shown to trigger a strong T-cell activation against the B-cell lymphoma, as measured by proliferative response and cytokine secretion. However, PCL-modification of DC pulsed with tumor extract added a dramatic increase to their immunogenic potential as compared with unmodified DC-pulsed with tumor antigen (Example 17 and Fig. 8).

It has been determined that the 2′, 3′-nucleoside and nucleotide dialdehydes such as 2′, 3′-adenosine dialdehyde (AdA) act as chemical crosslinkers of cell surface proteins in the PCL process through the action of providing a linkage between newly exposed proteins, as well as anchoring these proteins to the

cell membrane. It has also been determined that the above-described preincubation reaction conditions using, for example, 10 mM of the dialdehyde AdA provided for a large excess of the crosslinker relative to the number of potential targets for crosslinking within the cell membrane. It was further determined that under the improved PCL conditions including preincubation in crosslinker, the AdA molecules that were not involved in active crosslinking of cell membrane structures could react with membrane proteins (or proteins and peptides in general) through a single aldehyde moiety as monoaldehydes, thereby leaving the remaining aldehyde moiety free, i.e., a pendant aldehyde, e.g., (2′, 3′-adenosine monoaldehyde or AmA). In accordance with this determination, preincubation with dialdehyde allowed the cell membrane to be effectively derivatized with a monoaldehyde molecule, e.g., AmA, which was ultimately capable of forming a reversible chemical covalent bond (i.e., a Schiff base) with a $CD4^+$ T cell. The chemical reaction is depicted, for example, as follows:

(*immunogen, e.g., tumor cell*)-CH=O+H_2N-(*T cell*) ↔ (*immunogen, e.g., tumor cell*)-CH=N-(*T cell*).

Thus, in accordance with various aspects of the invention, dialdehyde can function as both a crosslinker of cell membrane components and as a reagent for monoaldehyde derivatization of cellular surfaces. This newly determined function of dialdehydes is achieved based upon the generation of two monoaldehyde moieties following the reaction of a cell (i.e., a cell surface protein or antigen (Ag)) with dialdehyde. By means of one of its aldehyde groups, the dialdehyde can form a reversible chemical linkage with membrane protein, peptide or carbohydrate, and by means of its other pendant aldehyde group, the dialdehyde ultimately can also provide a costimulatory or immunopotentiating linkage and recognition between a monoaldehyde (e.g., AmA) on the surface of a modified tumor cell and the amine group of cell surface protein on a T cell, for example. Accordingly, the invention encompasses the use of the monoaldehyde generated on the cell surface after treatment with dialdehyde, as a linking molecule for reaction with other biological molecules (e.g., cytokines, adjuvants, lymphokines) for slow or prolonged release of the attached molecule at a vaccination or immunization site.

The preincubation permits the formation of the critical concentration of monoaldehyde n the cell surface (i.e., not involved in intramembrane crosslinking) to provide a costimulatory signal to lymphocytes enhance and augment immunogenicity.

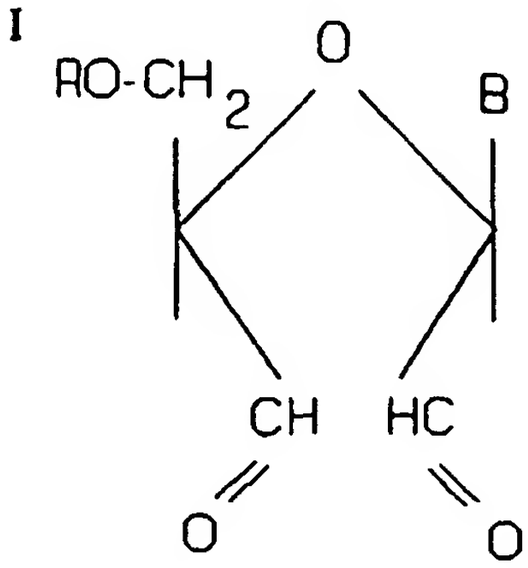
Accordingly and as described, the dialdehyde crosslinking agent employed as a protein crosslinker in the improved method of PCL modification of cells also functions as a general reagent for derivatizing proteins, thereby yielding a preincubated and PCL-modified cellular immunogen in which a dialdehyde with which the cells are reacted has one of its aldehyde groups capable of interacting with lysine groups of a second molecule such as an antigen, e.g., a protein antigen, or a carbohydrate antigen, so as to form one monoaldehyde linkage with antigen, thereby leaving the other pendant monoaldehyde group capable of interacting with another suitable molecule, for example, a protein on the surface of a cell, a nonmembrane-bound cytokine or peptide or portion thereof, or another immunostimulatory molecule. Such an immunogen is able to stimulate the immune response, in all likelihood, by functioning as a costimulatory or immunopotentiating immunogen wherein the dialdehyde may act as an adjuvant for increasing the immune response.

In preparing immunogens by means of PCL modification following preincubation with dialdehyde, tumor cells or infected cells are exposed to the action of a chemical crosslinking agent, which is a dialdehyde, preferably a 2′, 3′-nucleoside or nucleotide dialdehyde, which is referred to herein as a "crosslinker" or "crosslinking agent" or "crosslinking compound". In addition, the cells are subjected to a hydrostatic pressure in the range of from 800 to 1400 atmospheres, preferably about 1000 to about 1200 atm. Surprisingly, pressure of above about 1400-1600 atm or greater yields an immunogen having a far inferior anti-cancer immunization potency. The application and release of pressure is preferably gradual, e.g., over a period of 5 to 15 minutes. Immunogenicity of the modified cells is even further augmented if cells are exposed to crosslinking agent and to hydrostatic pressure at the same time.

The preferred crosslinking agent is a 2′, 3′-dialdehyde of a natural

nucleotide or nucleoside, since non-naturally occurring, i.e. synthetic, nucleosides r nucleotides are very often highly toxic. Such crosslinking agents are represented by the following formula I:

I

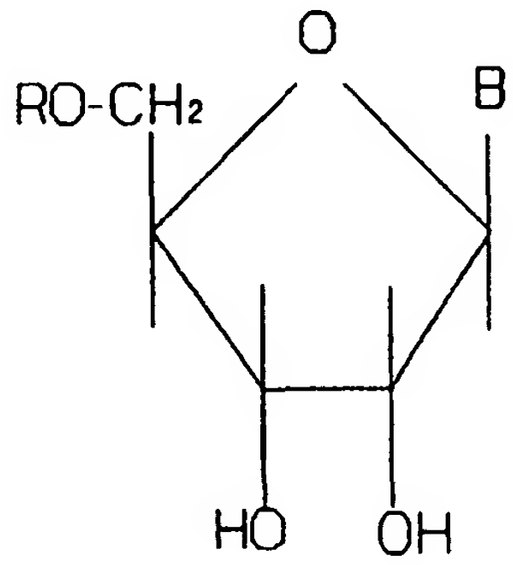


;

wherein, R is H, or a mono-, di- or tri-phosphate group, and
B is a nucleotide base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil. Specific, but non-limiting examples of these types of crosslinking agents are 2′, 3′-adenosine dialdehyde (AdA) and 2′, 3′-adenosine monophosphate dialdehyde (AMPdA).

The compound of formula I may be prepared by reacting a nucleoside or a nucleotide of the following formula II with an oxidizing agent, e.g. an alkali periodate:

II



wherein R and B have the meanings given above for formula I.

The improved PCL modification process is performed by preincubating isolated tumor cells or infected cells, and the like, with 2', 3'-nucleoside or nucleotide dialdehyde crosslinking agent in the range of about 1 mM to about 40 mM, preferably about 5 mM to 30 mM, and more preferably about 10 mM to about 20 mM, for a period of about 15 to 120 minutes, preferably about 20 to 60 minutes and more preferably for about 30 minutes, preferably at room temperature, i.e., about 25°C. A dialdehyde concentration of 10 mM for 30 minutes at room temperature was found to be especially effective for the preincubation of cells. Thereafter, cells were PCL modified as described herein and in Example 2 by exposure to dialdehyde crosslinker (e.g., 10 mM) and hydrostatic pressure (e.g., 1200 atm), with exposure to dialdehyde crosslinker and to pressure preferably being carried out at the same time at room temperature. It is noted that optimal immunogens are generated from the improved PCL method when cell viability following preincubation and PCL modification is greater than or equal to about 65 to 75%, as determined by Trypan Blue dye exclusion.

Another embodiment of the invention encompasses in general the formulation of compositions and products comprising dialdehyde as costimulatory adjuvanting molecules linked to antigens via one aldehyde group and having a pendant aldehyde group for chemical interaction with other suitable molecules, as described herein. Similar to the cellular immunogens that have been preincubated with dialdehyde, and crosslinked and pressure treated thereafter, in the compositions comprising dialdehyde and antigen encompassed by the invention, the pendant monoaldehyde moiety, which is unreacted with antigen in the composition, serves as a linker for attachment of other biological response molecules, e.g., cytokines, or peptides thereof, which may than act as slow or prolonged release adjuvanting agents at the site of vaccination or immunization, as further described hereinbelow. The compositions comprising dialdehydes reacted with antigenic protein or peptides or carbohydrate (either on cell surfaces or isolated antigens, preferably isolated antigens) provide an immunogen with enhanced or increased antigenic specificity and function. In a related aspect, the compositions of the invention provide a controlled immunostimulatory signal to activated T cells to

maximize the activation of responder T cells in a T_H1 mode. The compositions may be used both therapeutically and prophylactically.

Another embodiment of the invention comprises a dialdehyde component reacted with an antigenic component, e.g., a protein, a peptide, or a portion thereof, or a carbohydrate, for use in a vaccine or immunogenic formulation to afford specific immunological protection against the antigen as well as adjuvanting/costimulatory functions afforded by the monoaldehydic reacting groups generated by the dialdehyde. The antigen component may be considered to be "modified" via its chemical interaction with dialdehyde; thus, the composition can be considered to contain a "modified antigen". The resulting dialdehyde and antigen composition comprises a compound having one monoaldehyde group of the dialdehyde component attached to the antigenic component and a pendant monoaldehyde group of the dialdehyde component available for complexing with cell surface protein amine groups or the amine groups of non cell-surface-bound protein molecules.

The modified antigen and a pendant aldehyde group can be schematically depicted as follows: A-X-A + Immunogen ⇝ Immunogen-mA-X-mA; wherein A-X-A represents the dialdehyde that chemically reacts with an immunogen; Immunogen represents an antigen, either cellular or noncellular to which the dialdehyde is chemically complexed; mA-X-mA represents the monoaldehyde group of the dialdehyde which chemically reacts with immunogen, and the pendant monoaldehyde group that is capable of complexing with another protein or carbohydrate molecule. In the composition, one of the monoaldehydes derived from the dialdehyde is coupled to Ag or immunogen, while the other pendant monoaldehyde is free to combine with the appropriate chemical groups (e.g., amine groups) on other protein or carbohydrate structures. As indicated, antigens, such as proteins or carbohydrates, that are suitable for interaction with the pendant aldehyde group of the dialdehyde may be present on the surface of cells, or they may be unbound to cell membranes.

An advantage of the use of dialdehydes, for example, the 2′, 3′-nucleoside and nucleotide dialdehydes, and in particular, AdA, as costimulatory

adjuvanting molecules in the methods and compositions described herein is that the dialdehydes are operative and effective over an unexpectedly wide range of concentrations (e.g., from nanomolar to millimolar concentrations) as demonstrated by *in vitro* sensitization experiments. (Fig. 7).

Further, the present invention provides a number of other advantages to the art for increasing and enhancing the immune response to foreign or non-self antigens, namely, eliciting the presentation of immunologically important molecules on the cell surface for enhanced recognition by cells of the immune system; generating costimulatory molecules for T cells in a T_H1 mode, which is crucial for the cytotoxic response to eradicate tumor and infected cells; and providing anchors for the attachment of biological response modifiers to control environmental events at the site of immunization. The invention also addresses a formidable problem in the art concerning ways in which to stimulate, increase, and improve a host animal's immune response to tumor cells or otherwise transformed, infected, or foreign cells, by providing modified immunogenic cells which present tumor and foreign antigens to a host in the context of MHC (or HLA) molecules for appropriate recognition and destruction by the effector cells of the immune system. The modified cells, or membranes or proteins derived from the cells, are used either prophylactically or therapeutically as immunogens or in immunogenic preparations to immunize both naïve animals, including humans, as well as to treat tumored, infected, or diseased animals, including humans, which have previously encountered the tumor or foreign antigens. The immunogens containing antigen-bound monoaldehyde and free monoaldehyde resulting from the exposure of antigen to dialdehyde are further able to enhance a recipient's immune response via costimulatory or adjuvanting functions.

Preparation of the dialdehyde and cellular or noncellular immunogen/antigen composition comprises mixing and incubating the immunogen with dialdehyde at a concentration of between about 1 mM to 40 mM, preferably about 5 mM to 30 mM, and more preferably about 10 mM to 20 mM, for a period of about 15 to 120 minutes, preferably about 20 to 60 minutes and more preferably for about 30 minutes, preferably at room temperature, i.e., about 25°C. A

dialdehyde concentration of 10 mM for 30 minutes at room temperature is effective for saturating the available sites of the immunogen, thereby allowing for the binding of a monoaldehyde group of the dialdehyde molecule to the immunogen and for providing a free monoaldehyde group for binding to other cell structures, or to proteins, peptides, carbohydrates, and the like.

The dialdehyde and antigen compositions of the invention may be formulated into a pharmaceutically acceptable preparation comprising suitable carrier, excipient, or buffer and injected into a mammal in an amount effective to restore immune function to normal or near-normal levels or to increase the immune response to the particular antigen above normal levels to control, reduce, or eradicate a tumor or infection. The carrier is typically a conventional carrier for an injectable vaccine. The dialdehyde and antigen composition is used in an aqueous diluent which may also contain other soluble or particulate antigens, either alone or associated with a lipid carrier. The dialdehyde and antigen composition may be incorporated into water-in-oil emulsions, in liposomes, or in vehicles containing additional immunostimulatory or adjuvanting elements such as muramyl dipeptide for release at the site of administration, if such additional elements are necessary or desired.

The vaccine formulation comprises the antigenic component and the dialdehyde component, which may be considered to afford adjuvanting properties to the formulation. As a general guide, suitable amounts of vaccine formulation for injection into a host, including human patients, are from about 50 μl to about 5 ml, preferably about 100 μl to about 2 ml, more preferably about 200 μl to about 500 μl. The smaller amounts are generally more suitable for subcutaneous or intradermal routes of administration, while the larger amounts are generally more suitable for intramuscular routes of administration.

The dialdehyde and antigen components of the composition can be mixed prior to immunization, and then injected into a host or mammalian recipient, or each component may be injected independently at the site of immunization to provide the dialdehyde and antigen-containing immunogen, which is capable of stimulating and/or augmenting the recipient's immune response to the specific

antigen, essentially *in situ* at the inoculation site.

In another embodiment, the dialdehyde and antigen composition (i.e., the "modified antigen composition") may be "further modified" by reacting the unreacted pendant aldehyde group of the dialdehyde with an immunostimulatory, adjuvanting, or immunopotentiating compound. In this way, the composition can be made more highly immunogenic or can afford further adjuvanting or immunostimulatory properties after immunization in a recipient host. The modified composition can also be formulated into a pharmaceutically acceptable preparation comprising suitable carrier, excipient, or buffer and injected into a mammal, including humans, to increase the immune response to antigen. It will be appreciated by those skilled in the art that the dialdehyde and antigen composition may be admixed with other dialdehyde and antigen compositions to form a multivalent adjuvanting immunogen in which various antigens or elements thereof are combined. Alternatively, the dialdehyde molecule may be linked to two antigen molecules or elements thereof, via each of its aldehyde groups, or to two adjuvant molecules, e.g., lymphokines or cytokines, and used and administered separately or combined with other dialdehyde and antigen, or other dialdehyde, antigen, and adjuvant-containing compositions of the invention.

Examples of dialdehydes suitable for use in the compositions of the invention, include, but are not limited to, 2′, 3′-nucleoside or nucleotide dialdehydes, as described hereinabove, particularly, 2′, 3′-adenosine dialdehyde and 2′, 3′-adenosine monophosphate dialdehyde, malonic dialdehyde, and glutaric dialdehyde, with 2′, 3′-nucleoside or nucleotide dialdehydes being preferred. Those having skill in the art will be aware of other suitable or desired dialdehydes to use. Examples of antigenic components suitable for use in the compositions include, but are not limited to, any molecular structure that is antigenic and which contains amino groups, or can be induced, treated, or modified to contain amino groups, and which can be reacted with dialdehyde, such as proteins, polypeptides, peptides, and carbohydrate antigens. The antigens may be tumor or cancer antigens or peptides, antigenic proteins or components of bacteria, RNA and DNA viruses, particularly envelope polypeptides for enveloped viruses, parasites,

protozoa, mycoplasma, fungi, and yeast.

More particularly, examples of cancer or tumor antigens which may be used include those of various origins, for example, those derived from pancreatic tumors, ovarian tumors, cervical tumors, melanomas, sarcomas, breast cancers, gastric cancers, colon cancers, lung cancers, cancers of the head and neck, brain tumors, laryngeal cancers, rectal cancers, small lung carcinomas, non-small lung carcinomas, renal cancers, epitheliomas, bladder cancers, prostate cancers, lymphomas, non-Hodgkin's lymphoma, and leukemias.

As a further example, the antigen for formulation in the antigen and dialdehyde composition may be a protein or peptide that is synthetically or recombinantly produced, and isolated and purified as required prior to reacting with dialdehyde. The antigen may be a tumor associated antigen (TAA), or portion thereof, for which the gene has been cloned and the nucleic acid and amino acid sequences determined. Examples of TAAs that may be used in the compositions include, but are not limited to, melanoma TAAs which include, but are not limited to, MART-1 (Kawakami et al., 1994, *J. Exp. Med.*, 180:347-352), MAGE-1, MAGE-3 (Gaugler et al., 1994, *J. Exp. Med.*, 179:921-930), GP-100, (Kawakami et al., 1994, *Proc. Nat'l. Acad. Sci. USA*, 91:6458-6462;), oncofetal antigens, CEA, TRP-1, P-15, the gp160 and rev proteins of HIV-1, the H1 antigen of influenza, the circumsporozoite protein from malaria, nucleoprotein from LCMV (Fynan, E., et al. 1995. *Proc. Natl. Acad. Sci. USA*, 90:11478; Wang, B., et al. 1993. *Proc. Natl. Acad. Sci. USA*, 90:4156; Sedegah, M., et al. 1995. *Proc. Natl. Acad. Sci. USA*, 91:9866) and tyrosinase (Brichard et al., 1993, J. Exp. Med. 178:489), and the like. The TAAs can also be CA-19-A (pancreatic cancer), CA-125 (ovarian cancer), PSA (prostate cancer), erb-2, (breast cancer), HER-2neu (breast and ovarian cancer), CA-171A and the like (Boon et al., 1994, Ann. Rev. Immunol. 12:337). However, the antigens for use in the dialdehyde and antigen composition are not intended to be limited to the above listed TAAs.

Viral antigens for use in the invention include subunit antigens from influenza virus, from human immunodeficiency virus, e.g., gp 120 and gp 160, and from hepatitis viruses. Attenuated, e.g., heat-killed or detergent-killed, whole

organism vaccines are suitable for use with the dialdehyde component in the formulation of more highly immunogenic vaccine preparations. Other examples of viral antigens include antigens from cytomegalovirus, polio virus, respiratory syncytial virus, herpes simplex virus, human papilloma virus, Epstein Barr virus, adenovirus, rhinovirus. Animal viruses whose antigens are suitable for use in the compositions of the invention include rabies virus, foot and mouth disease virus, equine flu, equine encephalitis, feline immunodeficiency and feline leukemia viruses. Bacterial antigens for use in the compositions of the invention include among the non-limiting examples, B. pertussis, from which the following antigenic components may be used with dialdehydes as adjuvanting compounds to provide effective vaccines: pertussis toxin, filamentous haemagglutinin, and pertactin, P69; C. tetani, N. meningitidis, N. gonorrhea, S. aureus, S. pneumoniae, H. influenzae, C. diptheriae, P. aeruginosa, V. cholerae, E. coli, B. subtilis, Campylobacter jejuni and Campylobacter pylori. Parasites (e.g., P. falciparum; L. major) and yeast (e.g., Aspergillus; C. albicans and the like) or fungi or algae also offer protein or carbohydrate antigenic components for use with dialdehydes in the compositions of the invention. Pathogenic protozoans having antigenic components that may be used include malaria, Babesia, Schistosoma, Toxiplasma, Toxocara canis, and the like. Protein or antigenic components from these and other viruses and bacteria may be used in the dialdehyde-containing formulations of the invention to provide vaccines and immunogenic preparations against pathogenic infections.

Examples of immunostimulatory, adjuvanting, or immunopotentiating molecules that can be reacted with the pendant monoaldehyde group in the above-described compositions of the invention include, but are not limited to, cytokines such as interleukins IL-1 (i.e., IL-1α and IL-1ß) to IL-16; interferons such as alpha, beta or gamma interferon; hematopoietic cell growth and proliferation factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), human growth hormone (hGH); TNF-ß and TNF-α; RANTES (Regulated upon Activation, Normal T Expressed and presumably Secreted cytokines, Promega, G5661), and the like; leukotrienes; T cell proliferation

factors, e.g., OKT3, and the like, and as described hereinbelow as adjuvants. Other such immunostimulatory molecules are B7-1, B7-2, ICAM-1, ICAM-2, LFA-1, LFA-3, CD72 and the like. Preferred cytokines include IL-2, IL-12, IFN-γ (gamma interferon), and TNF-ß. Examples of chemokines that may also be used in the present invention include, but are not limited to, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MBSA, MIP-1α, MIP-1B, and the like. It is to be understood that combinations and mixtures of the foregoing examples of immunostimulatory, adjuvants, or immunopotentiating molecules may be attached to an antigen or immunogen through the dialdehyde of the compositions in accordance with the invention.

The costimulatory molecules of the B7 family (namely B7-1, B7-2, and possibly B7-3) represent a more recently discovered, but important group of cell regulatory, immunostimulatory or accessory molecules. B7-1 and B7-2 are both members of the Ig gene superfamily. These molecules are present on macrophages, dendritic cells, and monocytes, i.e., antigen presenting cells (APC). If a lymphocyte encounters an antigen alone, without costimulation by B7-1 or an equivalent stimulatory event or signal, it will respond with either anergy or apoptosis (programmed cell death); if the costimulatory signal is provided, it will respond with clonal expansion against the target antigen. In general, no significant amplification of the immune response against a given antigen occurs without costimulation. It will be appreciated that the dialdehyde component of the present invention is capable of providing costimulatory signaling via its monoaldehyde functionality and its interaction(s) with cell surface molecules, particularly those on T cells, more particularly T cells of the T_H1 subclass. It is noted that the B7-2 costimulatory molecule is generally associated with the stimulation of a T_H2 type immune response; thus, B7-2 linked via the pendant monoaldehyde in the dialdehyde and antigen composition could stimulate T cells of the T_H2 subclass, if necessary or desired.

It is to be understood that the compositions of the invention may be used alone or in combination with other therapeutic agents for the treatment of cancers and pathogenic diseases caused by the above infections and conditions.

Such combination therapies may comprise the administration of at least one composition of the invention or a physiologically functional derivative thereof, and at least one other pharmacologically active ingredient. The pharmacologically active ingredient(s) may be administered separately or together. When administered separately, this may occur at the same time or sequentially in any order. Based on conventional methods and knowledge, those skilled in the art will select the amounts of the active ingredient(s) and pharmacologically active agent(s) and the relative timing, schedule, and mode of administration to achieve the desired combination therapeutic effect. Preferably, such combination therapy comprises the administration of at least one dialdehyde and antigen composition of the invention and another therapeutic agent. Nonlimiting examples of therapeutic agents are those employed, for example, in the treatment of HIV or hepatitis virus infections or associated conditions, such as the 2′, 3′-dideoxynucleosides, e.g., 2′, 3′-dideoxycytidine, 2′, 3′-dideoxyadenosine, and 2′, 3′-dideoxyinosine; 3′-azido-3′-deoxythymidine (zidovudine); acyclic nucleosides, e.g., acyclovir; interferons, e.g., α-interferon; TAT inhibitors; protease inhibitors; ribavirin; nucleoside transport inhibitors, and the like.

As described hereinabove, the dialdehyde component of the invention can be considered to serve as adjuvant in combination with antigen in a vaccine or immunogenic preparation. It is to be understood that while the components of the compositions may be administered as purified or commercially available preparations, either recombinantly or synthetically produced, isolated, and purified, they can also be prepared in a pharmaceutical formulation. Accordingly, a vaccine or immunogenic preparation may be formulated comprising the dialdehyde component and the antigen component in admixture with one or more acceptable carriers and other therapeutic agents, if desired. Acceptable carriers are those which are compatible with other ingredients in the formulation and not deleterious to the host or patient. As mentioned above, the dialdehyde and the antigen components may be prepared in suitable buffer or diluent and admixed or combined prior to or at the time of administration as immunogen. The route of administration may include parenteral, including subcutaneous, intradermal,

intramuscular, and intravenous; intranasal, oral, rectal, inhalation, or topical. Effective amounts of the compounds in the composition and an effective dose of the composition will depend upon a number of factors known to those having skill in the art, including the nature of the recipient host, i.e., human, non-human primates, other mammalian species; the type and size of the host; the severity of the disease, cancer, or condition being treated; the route of administration; and general and specific responses by the patient. For convenience, the total daily dosage may be divided and administered in portions throughout the day, if desired.

An effective dose of the compositions of the invention via the above-mentioned routes of administration for human use for the purposes of vaccines and to provide costimulatory signals can be conventionally determined by the practitioner. Effective doses and amounts will depend on a number of factors mentioned hereinabove, known to those in the art. As a general guide, doses may range from 0.01 to 100 g/M^2 in a human recipient, preferably in the range of 0.05 to 50 g/M^2, more preferably in the range of 1 to 10 or 20 g/M^2. An exemplary dose range is about 1 to 3 g/M^2, for example, for two to five, preferably three, days per month. Of course, determination of the proper dosage and administration schedule for a particular situation is within the skill of the medical practitioner, and significantly lower doses (i.e., in the ng and mg ranges) may be administered, given the potency and large effective concentration range of action of the dialdehyde as adjuvant or immunostimulator. The effective dose as desired may be administered at multiple appropriate intervals throughout the day, e.g., as between two and five sub-doses, or for a period of several days monthly.

The preparation and administration of pharmaceutically acceptable formulations are known in the art. Formulations containing a daily dose or unit daily subdose as described or known to those in the art, or an appropriate fraction thereof, of the compounds of the invention, or physiologically acceptable salts thereof, are preferred unit dose formulations.

Since parenterally-administered drugs may have much greater bioavailability than the oral forms, parenteral doses may be only a fraction of what would be given orally. Pharmaceutical preparations containing the dialdehyde and

antigen-containing compositions as described and conventional pharmaceutical carriers may be employed in unit dosage forms such as solids or liquids. The methods for preparing unit dosage formulations are well known and practiced in the art of pharmacy. All of the methods involve bringing into association the compounds of the compositions, i.e., the active ingredients, with the carrier which comprises one or more accessory ingredients. Generally, the formulations are prepared by uniformly and intimately bringing into association the active ingredient(s) with liquid carriers or finely divided solid carriers, or both, and then, if necessary, shaping the product into the desired formulation.

For example, solid form preparations include, for example, tablets, pills, capsules, powders, dispersible granules, cachets, and suppositories. Liquid form preparations include isotonic solutions, suspensions, or elixirs for oral administration or liquid solutions, suspensions, and emulsions for parenteral use. Suspensions may be in an aqueous or non-aqueous liquid, as an oil-in-water liquid emulsion, or as a water-in-oil liquid emulsion. The active ingredient(s) may be presented as a bolus, electuary, or paste. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, for example, packeted tablets, capsules, and powders in vials or ampules. The unit dosage form can also be a capsule, cachet, or tablet itself, or it can be the appropriate number of any of these in packaged form. Tablets contain the active ingredient in admixture with non-toxic, pharmaceutically-acceptable excipients which are suitable for the manufacture of tablets. These excipients may be, for example, inert diluents, for example, calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating or disintegrating agents, for example, maize starch or alginic acid; binding agents, for example, starch, gelatin, or acacia; and lubricating agents, for example, magnesium stearate or stearic acid. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract, and thereby provide a sustained action for a longer period of time.

The composition may be formed by dispersing the components in a suitable pharmaceutically-acceptable liquid or solution such as sterile physiological

saline or other injectable aqueous liquids. The composition may be administered parenterally, including subcutaneous, intravenous, intramuscular, or intrasternal routes of injection or infusion techniques, although conventionally via subcutaneous or intramuscular routes of injection. For parenteral administration, the composition is in sterile solution or suspension or may be emulsified in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives and material for rendering the solution or suspension isotonic with body fluids (i.e. blood) of the recipient. Such formulations may be used in unit-dose or in multi-dose containers (e.g., sealed ampules and vials) for convenience. Excipients suitable for use are water, phosphate buffered saline, pH 7.4, 0.15 M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. The sterile solutions for injection may also contain antioxidants, buffers, and bacteriostatic agents. The unit or multi-dose formulations may be stored in a freeze-dried or lyophilized condition requiring only the addition of sterile liquid carrier, e.g., water, for dissolving the injectable material prior to use. In addition, extemporaneous injection solutions may be prepared from sterile powders, granules, and tablets of the kind as described herein.

If oral administration is desirable or required, the composition may be presented as a draught in water or in a syrup, in capsules, cachets, boluses, or tablets, as an aqueous or oleaginous solution or suspension, or a suspension in a syrup. Such suspensions optionally may include suspending agents, or may be presented as an oil-in-water or water-in-oil emulsion. Where desirable or necessary, flavoring, sweetening, preserving, emulsifying, or thickening agents may be included in the formulation. Examples of sweetening agents are glycerol, sorbitol, or sucrose. Such formulations may also contain a demulcent and coloring agents. Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate, or kaolin, or as soft gelatin capsules, wherein the active ingredient is mixed with an oil medium, for example, arachis oil, liquid paraffin, or olive oil. Additional formulations suitable for other modes

of administration such as suppositories may include binders and carriers, for example, polyalkylene glycols or triglycerides.

Tablets may contain the preparation as a powder or granules, for example, a lyophilized powder or granules optionally mixed with binders, lubricants, inert diluents, or surface-active or dispersing agents, and may be formed by compression or by mouling in inert liquid diluent. Such tablets may be optionally scored and/or coated. Capsules and cachets may contain the active compound(s) alone or in admixture with one or more accessory ingredients. Capsules may also contain the active ingredients in aqueous or oleaginous solution, suspension, or emulsion, optionally in association with accessory ingredients. Presented in unit dosage form, each dose may be conveniently contained in, but is not limited to being contained in, volumes of from about 0.1 mL to about 1.0 mL, preferably about 0.5 mL.

Aqueous suspensions contain the active ingredients in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example, sodium carboxymethyl cellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth, and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example, polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example, heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol, for example, polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example, polyoxyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example, ethyl or n-propyl ρ-hydroxy benzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose, saccharin, or sodium or calcium cyclamate.

Suppository formulations for rectal administration may be presented

with the usual carriers such as cocoa butter. It is also to be understood that the compounds of the invention or physiologically acceptable salts thereof may be presented as slow release or depot formulations as known in the art from which the active ingredient is released over a prolonged period of time, once the formulation is in place in the body of the recipient, e.g., transdermal patch or subdermal injectables, and the like, as known in the art.

With regard to PCL-modified cellular immunogens produced by the improved method of the invention, the improved method generates cellular immunogens having surface proteins crosslinked by dialdehyde, as well as monoaldehydic functionalities on the cell surface suitable for interacting in an costimulatory capacity with immune cells, for example, or in an adjuvanting capacity. The dialdehyde-preincubated and PCL-modified cellular immunogen may consist of whole modified tumor cells, membranes derived from such cells, as well as protein obtained from such cells or membranes, which substantially retain the capability of the modified tumor cells to induce the anti-tumor immune response. As described, the immunogen may be cells infected with bacteria, parasites, protozoans, yeast, or fungi. Also encompassed by the invention is direct PCL-treatment of bacterial cells, and the like, for enhancing the presentation of antigen of the pathogen to the cells of the immune system to increase the specific immune response against the invading pathogenic microorganisms. Such direct PCL modification of isolated pathogenic cells may serve to present to the cells of the immune system previously unexpressed antigens, or to increase the relative numbers of antigens, which are normally expressed at low or undetectable levels, on the surfaces of the pathogenic cells.

Preferably, following the preincubation and PCL treatment, the modified cells, particularly tumor cells, are exposed to high intensity radiation in order to destroy their genetic material. This is particularly important where the whole modified tumor cells are used for immunization, but may not necessarily be required where said immunogen consists of membrane preparations or membrane-derived proteins.

Where the desired immunogen consists of the whole modified tumor

cells, the product of the above process may be used *per se* or after several purification treatments, e.g., consisting of centrifugation and removal of the supernatant. Where the preparation consists of membranes of such modified tumor cells, the modified tumor cells are subjected to further treatment in which the cells are disrupted, e.g. by exposure to a hypotonic medium or by sonication, and then the membrane fragments are collected e.g. by centrifugation in a sucrose gradient, as known in the art and as generally described in Example 13. Where the desired immunogen consists of protein material, the whole modified cells or the plasma membranes are subjected to further treatment, for example, dissolving or solubilizing the membranes using detergents, separating the proteinaceous material by one of various methods conventionally known, e.g., gel filtration, and then determining which of the separated proteins and/or proteinaceous material fragments possesses the desired immunogenicity.

The immunogen may be used for the immunization of cancer patients against their tumor or may be used for the sensitization and proliferation of immune cells *in vitro* (i.e., in *in vitro* sensitization or "IVS" assays). For immunization, the immunogen may be injected into a patient together with a pharmaceutically acceptable carrier or adjuvant in an amount sufficient to achieve an anti-cancer or tumor immune response. For *in vitro* sensitization, peripheral blood mononuclear cells, including immune cells, e.g., leukocytes or lymphocytes, are withdrawn from the patient by known methods and are then cultured together with the immunogen until a population of such immune cells reactive against said immunogen is obtained (see Example 10). Such a stimulated population of immune cells may then be reinjected into a cancer patient in order to treat his/her tumor.

While the immunization of patients in accordance with the present invention can be performed by employing an allogeneic immunogen, it is preferably performed by employing an autologous immunogen. The use of an autologous immunogen provides significant advantages in that the immune response which occurs is primarily directed against the neo-antigen of the tumor. When an allogeneic immunogenic preparation is used, the resulting immune response will be against all of the "non-self" or foreign antigens of such an immunogen. The use of

an autologous immunogen has the further advantage in that the neo-antigens associated with a specific tumor may differ from one patient to another. However, allogeneic immunogens also provide significant immune responses against PCL-modified allogeneic cells in humans.

Methods in which an autologous PCL modified cell preparation is used to prepare an immunogen comprise: withdrawing a tumor growth or infected cell population from a patient by biopsy or surgery; dissociating intact tumor, cells or infected cells, if necessary, by mechanical or enzymatic means; dispersing the cells in a medium; preincubating the cells with 2′, 3′-nucleoside or nucleotide dialdehyde, e.g., 2′, 3′-adenosine dialdehyde (AdA); exposing the cells to the 2′, 3′-nucleoside or nucleotide dialdehyde, e.g., 2′, 3′-adenosine dialdehyde (AdA), and to hydrostatic pressure in a concentration and for a time sufficient to modify proteins in the cells' plasma membranes; preparing a tumor-specific immunogen derived from the preincubated and modified cells obtained; injecting the immunogen into the patient, whereby an anti-tumor immune response in the patient is induced. A preferred embodiment comprises exposing the cells to a hydrostatic pressure between about 800 and about 1400 atmospheres, preferably about 900 and about 1200 atmospheres, more preferably, 1000 or 1200 atmospheres, at the same time that the cells are exposed to the 2′, 3′-nucleoside or nucleotide dialdehyde crosslinker, at a concentration of about 5 to 40 mM, preferably 10 to 20 mM. As an alternative, the method may involve the step of using the modified tumor or infected cells in an *in vitro* sensitization assay with immune cells to generate stimulated, sensitized immune cells, i.e., leukocytes and lymphocytes, which will react against and ultimately destroy the tumor cells following injection *in vivo*. Another alternative of the method involves washing the cells following preincubation in dialdehyde and PCL modification, e.g., three times in HBSS, resuspending the cells in medium containing serum, e.g., human AB serum, and cryopreserving the cells for future use.

Where an allogeneic immunogen is used, an immunogen derived from modified tumor or infected cells obtained from a defined tumor or infected cell line or cell type may be used. In addition, modified tumor cells obtained from

the same tumor type from another source or donor individual may be used with equal success. Similarly, infected cells for PCL modification may be from another source or donor individual having the same infection.

When used as immunogens *in vivo*, the modified cells or cell preparations may include pharmaceutically acceptable carriers, excipients, or formulations, such as normal or buffered saline and the like. In addition, PCL-modified cell immunogens may further include non-classical adjuvants which are co-injected *in vivo* or are formulated into the immunogenic preparation or vaccine comprising PCL-modified cells, cell preparations, plasma membranes, to enhance the immune response. It is to be understood that these types of adjuvants can also be used in the above described compositions comprising dialdehydes and proteins. Nonlimiting examples of such non-classical adjuvants, or mixtures and combinations thereof, that can also be injected with PCL-modified cells or membranes include human growth hormone (hGH), hematopoietic cell stimulating factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), and the like, Bacillus calmette guerin or BCG, saponins, T cell stimulating or activating factors, such as OKT3, TNF-α, and the like, interleukins, e.g., IL-1 to IL-16, and interferons, e.g., alpha, beta, and gamma interferons. Those skilled in the art will appreciate that the use of such non-classical adjuvants, e.g., GM-CSF or hGH, will ultimately allow conservation of the number of PCL-treated cells that are used as immunogens in accordance with the invention. In particular, the use of adjuvants is likely to allow a reduction in the number of tumor or infected cells required for use in immunogenic preparations, especially in combination with one or more adjuvants in amounts determined to provide an effective and enhanced immune response, for example, as determined by DTH assay. For example, the use of adjuvant such as GM-CSF (100 μg) in conjunction with cellular immunogen (e.g., $3x10^6$ cells) on day 1, and subsequent injections of GM-CSF (75 μg) alone on days 2 and 3, allow a reduction in the number of immunogen cells required for use in a subsequent immunization by as much as one third to one half the number of cells originally used, without sacrificing vaccine potency.

It will also be understood by those skilled in the art that combinations or mixtures of adjuvants (e.g., GM-CSF together with hGH, as a nonlimiting example) may be used in conjunction with PCL-based immunogens to augment the immune response. In addition, for those compounds which may affect cells by different biochemical or biological pathways, for example, GM-CSF and hGH, the use of a combination of adjuvants may result in a synergistic effect, thereby providing a more pronounced augmentation of the immune response to the immunizing preparation. Non-classical adjuvants may be administered in doses which one skilled in the art may determine by known methods and protocols to be efficacious in augmenting the immune response. As an exemplary guide, non-classical adjuvants may be administered in the range of about 1 μg to 1 mg per injection, preferably about 100 μg to about 400 μg, and more preferably about 50 μg to 200 μg. GM-CSF in particular may be administered in the range of about 1 to 500 μg per injection, preferably about 100 to 200 μg per injection, and more preferably about 50 to 100 μg per injection. As another example, human growth hormone may be administered in the range of about 0.01 to 3.0 mg/kg of body weight, preferably about 0.05 to 1.25 mg/kg.

As will be apparent to those of skill in the art, the doses or amounts of modified cells for use as immunogens will be determined by the skilled practitioner and will depend upon the types of cells used, the tumor load, if applicable, the extent and severity of infection and the like, and the overall condition and immune status of the patient. As a general guide, an immunogenic formulation is prepared comprising PCL-modified cells in the range of at least about 1.0×10^5 to 1.0×10^6 or more (e.g., to about 20.0×10^6) per immunizing dose, in admixture with saline or other excipients known in the art. Although fewer and more than about 10^6 cells may be used, a suitable number of cells is about $1\text{-}2 \times 10^6$ for an immunizing dose. Those skilled in the art can routinely determine the appropriate cell number for an immunizing dose, depending on the type of tumor or infection under treatment. A greater number of modified cells formulated in the immunogenic preparation may increase or heighten an individual's immune response to his/her tumor or infection. Normal modes of

administration, e.g., intravenous, subcutaneous, intradermal, intramuscular, sublingual, intraperitoneal, percutaneous, intrathecal, intracutaneous, r enteral, may be used with the immunogenic compositions afforded by the invention; the preferred routes of immunization are intradermal and intravenous. In addition, local administration to the afflicted site may be accomplished through means known in the art, including injection and implantation.

As explained *supra*, the term PCL-modified as used herein means cells treated in accordance with the PCL methods of the invention. The terms tumor cell, transformed cell, cancer cell, and infected cell refer to cells which contain, display or present on their surfaces foreign protein or peptide antigens to the cells of the immune system, regardless of whether the presented antigens are autologous or allogeneic to the host. The term antigen can refer to a protein or peptide structure, molecule, complex, or component thereof, or a carbohydrate that is generally recognized as foreign, non-self, and immunogenic by cells of the immune system. The antigen may be present on the surface of a cell, or it may be unbound or unassociated with a cell surface. It will be appreciated by those in the art that an antigen may be an epitope or determinant comprised of a series of amino acid residues, e.g., comprising from about 3 to about 7 residues, or from about 5 to about 10 or more residues, that are recognized or bound by immune cells due to their particular configuration and/or conformation characteristics. The method of the invention encompasses cells which have in some way become distinct from normal cells due to genetic or *in vivo* events, or to exogenous events or agents, resulting in cancer cells, tumor cells, non-normal or non-self cells, or cells causing another type of pathogenic or disease condition in an animal, including humans.

In addition to and including the virus, pathogen, tumor and cancer types, the proteins, peptides, or carbohydrates of which have been described above as suitable for the antigen component of the dialdehyde and antigen compositions of the invention, nonlimiting examples of cancer or tumor cells and infected or transformed cells which may be preincubated with dialdehyde and modified by the improved PCL method and used in PCL vaccines of the invention are all types of

tumor or cancer cells of various origin, including, but not limited to, cells derived from pancreatic tumors, ovarian tumors, melanomas, sarcomas, breast tumors, colon cancers, lung cancers (e.g., mesotheliomas), small-cell lung carcinomas, non-small cell lung carcinomas, liver or renal cancers, bladder cancers, prostate cancers, tumors and cancers of hematopoietic origin (e.g., lymphomas and leukemias), and the like; as well as virally-infected cells of different origins, including those of the lymphoid and hematopoietic system, including virally-infected T and B cells, macrophages, neutrophils, and the like. Virus-infected cells include those containing DNA and RNA viruses which infect and/or reside in cells of various types and which may be oncogenic. Non-limiting examples of the types of virally-infected cells that are suitable for PCL modification and used in the invention are human immunodeficiency virus (HIV)-infected cells of various strains (e.g., HIV-1 and HIV-2), human T-lymphotrophic virus-infected cells, *Herpes simplex* virus-infected cells, Epstein-Barr virus-infected cells, Hepatitis A, B, C, D, and E-infected cells, and the like. The invention is not limited to cells at any particular stage in development. The various cell types may also be transformed, infected, or otherwise altered by a variety of endogenous or exogenous means, such as by genetic mutations or abnormalities, exposure to chemical or physical carcinogens, infection by viruses, intracellular bacteria, parasites, or other disease-causing agents, thereby resulting in cells, e.g., tumor cells or cancer cells or infected cells, that are transformed or morphologically changed away from their normal cell counterparts. Examples of the types of bacterially-infected cells for use in the invention include, but are not limited to cells infected with the mycobacteria that cause leprosy, *E. coli*-infected cells, *S. aureus*-infected cells, *Streptococcus*-infected cells, *Shigella dysenteriae*-infected cells, *Borrelia burgdorferi*-infected cells, *Bacillus tuberculosis*-infected cells, meningococcal-infected cells and the like. Non-limiting examples of types of parasites that can infect cells and that can be treated and used in accordance with the invention are malaria, leishmania, and schistosomes, and the like.

EXAMPLES

The examples as set forth herein are meant to exemplify the various

aspects of carrying out the invention and are not intended to limit the invention in any way.

Example 1

A. Cells and reagents

Cells of ARadLV 136, which is a radiation-induced leukemogenic variant of ARadLV, were maintained *in vitro* as described previously (Haran-Ghere et al., 1977, J. Immunol. 118:600).

B16-BL6 melanoma tumor cells syngeneic (i.e., autologous) to C57BL/6 mice were serially passaged in mice by subcutaneous inoculation of 2-5 x 10^5 cells in 1.0 mL Hanks Balanced Salt Solution (HBSS) or phosphate buffered saline (PBS) at physiological pH.

B. 2', 3'-Adenosine dialdehyde (AdA)

AdA, which is a biologically compatible chemical crosslinker, was synthesized by a modification of the procedure previously described (Hansske et al., 1974, Bioorg. Chem. 3:367). The procedure was as follows: Adenosine (Sigma Chemical Co., St. Louis, MO) and sodium metaperiodate (Fluka Chemie AG, Buchs. FRG) were mixed in 100 mL aqueous solution to a final concentration of 10 mM of each of these substances, stirred in the dark, and cooled with ice water for 1 hour, and then concentrated to 5 mL under vacuum at 30°C. The resulting concentrate was then incubated for 12 hours at 4°C and the crystalline product which was obtained was separated and found to be homogeneous in thin-layer chromatography (silica gel G plate, 0.2 mm thickness, Merck Darmstadt; running solvent; acetonitrile/water, 4:1 v/v, R_F 0.80). The crystals were filtered, washed three times with cold water and dried over silica gel in vacuum (12 mm Hg: 1.6 kPa).

The yield of the above preparation procedure was found to be approximately 90%. The obtained product had a melting point of 110°C and melting was accompanied by decomposition, this being in agreement with previous reports (Hansske et al., 1974, *supra*). The final AdA product as prepared and used in the PCL modification of the invention should be active in crosslinking membrane proteins in accordance with the invention, should be free of iodate

impurities, and should be soluble at the pH in which crosslinking is performed (i.e., about neutral) in accordance with the invention. Those skilled in the art will appreciate that the molar concentration of AdA can be determined by measuring the adenosine concentration or the dialdehyde concentration of the AdA preparation. It is noted, for example, that an AdA concentration of about 20 mM, as determined by measurement of the adenosine concentration of the AdA preparation is equivalent to an AdA concentration of about 10-13 mM, as determined by measurement of the dialdehyde concentration of the same AdA preparation. Accordingly, *in vitro* and *in vivo* results obtained using about 20 mM AdA as measured by its adenosine content and those obtained using about 10-13 mM AdA as measured by its dialdehyde content are essentially equivalent.

The 2′, 3′-nucleoside and nucleotide dialdehydes have several features which make them advantageous for use in the invention. These crosslinkers are biocompatible and are virtually non-toxic to cells when used in accordance with the invention; they are membrane impermeant; they have a slow rate of uptake into the cell, and thus are retained longer in the cell membrane where they can effect their crosslinking functions; they do not interfere with solubilizing plasma membranes prior to membrane isolation; they are non-immunogenic by themselves; they also possess a relatively long shelf life.

C. Batch to batch assessment of crosslinking capability of AdA

To assess the crosslinking capability of each preparation (batch) of dialdehyde AdA and to assess the batch to batch variation among preparations of dialdehyde, a functional assay was developed in which hen egg white lysozyme was incubated, or mixed, and reacted with AdA and a precipitate of polymerized lysozyme was formed. After centrifugation, the residual tryptophan fluorescence of the lysozyme (λ_{em} = 348 nm) in the supernatant was measured (emission intensity). In general, in performing the assay, the emission intensities of samples of lysozyme before and after reaction with dialdehyde were determined. If lysozyme remained uncrosslinked after incubation with dialdehyde, the emission intensity measurement of the solution was similar to the measurement determined before the addition of dialdehyde. Crosslinking of lysozyme by dialdehyde resulted

in a decrease in the emission intensity of the solution, dues to the formation of crosslinked precipitate. A predetermined range of acceptable crosslinking capacity of AdA with lysozyme was determined in which a maximum scale reading was determined for the egg white lysozyme solution at a particular wavelength. Preparations of AdA having a crosslinking capacity that fell within 2 standard deviations of the predetermined range were used for preincubation and in PCL experiments, and units of dialdehyde were normalized to the reading for lysozyme at pH 9. Based on these analyses, it was found that at 10 mM AdA, there was a large difference between active AdA samples and the adenosine control, while there was virtually no differences among the emission intensities of the three batches of AdA prepared and tested for use as (see Fig. 1). In this way, quality control checks were able to be routinely performed on preparations of AdA and the crosslinking function was able to be consistently evaluated as needed.

D. Evaluation of the binding kinetics of AdA

The kinetics of AdA binding was determined using approximately 1 x 10^7 B16-BL6 cells and a [^{3}H]-adenosine tracer present at a final concentration of 10 mM AdA. The B16-BL6 cells were prepared from freshly excised tumors in mice; however, B16 cells grown in culture may also be used. The cells were incubated in 10 mM AdA for various periods of time, and the amount of AdA bound was determined after washing the cells after incubation for the appropriate time period. All measurements were performed on a beta-counter in duplicate. In Fig. 2, the percent bound AdA was determined as a function of pH. Three pHs were used (pH 7.0; pH 7.5; and pH 8.0). The results showed that there was no difference in the rate of AdA binding to cell membranes in the physiological pH range. Overall, only about 1.5% of the AdA bound to the cells within two hours; this amount rose to 2% after 24 hours. Using 2% as the saturation point, the number of molecules of AdA per cell is in the range of 10^{10} to 10^{11}.

E. Determination of the percent occupancy of available cell surface binding sites for AdA

The determination of the percent occupancy of available binding sites for AdA on the cell surface was carried out using B16-BL6ex cells, 10 mM AdA

and a [^{3}H]-adenosine tracer present at a final concentration of 10 mM AdA, as described above. Fig. 3 shows that 50% binding occurs only after 27 minutes. Further, in the first three minutes of AdA incubation, about 10% of the AdA binds to available sites on the cells. This three minute incubation with AdA is unavoidable; however, the binding that occurs in this period is insignificant. The kinetic analysis of AdA binding demonstrates that the reaction is of first order kinetics and that the crosslinker reacts slowly with respect to the length of the incubation procedure (usually 1 hour), thus allowing sufficient time for the crosslinking of newly-exposed proteins during the application of pressure to the preincubated cells. These same experiments were carried out using higher concentrations of AdA (e.g., 20 mM to 40 mM) and virtually the same results were obtained.

Example 2

Procedures for preincubation of cells in dialdehyde crosslinker and PCL-modification of cells

After harvesting and obtaining suspensions of tumor cells as described in Examples 8 and 9 or obtaining infected cell, pathogenic cell, or even peripheral blood mononuclear cell (PBMC) preparations, the cells were subjected to dialdehyde preincubation and to PCL-modification as described hereinbelow. The viability of the cells before and after PCL treatment was assessed by trypan blue dye exclusion.

In this example, up to about 5×10^7 to 1×10^8 freshly harvested or previously cryopreserved cells (e.g., tumor cells, infected cells, PBMCs) were resuspended in HBSS or PBS, preferably sterile, in a 50 mL tube (Falcon, Becton Dickinson Labware, N.J.) containing 10 mM adenosine dialdehyde (AdA) and were incubated in this solution for 0 minutes to 1 hour at room temperature with occasional mixing. It was determined that a 20 to 40 minute, preferably a 30 minute, preincubation in dialdehyde was effective for achieving maximal presentation of monoaldehyde on the cell surface (see Table 1) and presentation of antigen for stimulation of immune cells. Preincubation of cells in dialdehyde for 20 to 30 minutes, and up to and including 1 hour, revealed the same level of

monoaldehyde and antigen presentation on the cell surface following PCL treatment. Less than 20 to 30 minutes of preincubation resulted in inconsistent and suboptimal crosslinking, modification, and monoaldehyde formation.

Following preincubation with dialdehyde, PCL treatment was carried out as follows: up to about 5 x 10^7 to 1 x 10^8 of the preincubated cells were suspended in sterile HBSS containing 10 mM AdA and were immediately exposed to 1,200 atmospheres of hydrostatic pressure for 15 minutes using a pressure bomb, as described below, or more preferably, a computer controlled pressure device (APP, Inc., Ithaca, NY). Following decompression the cells were preferably allowed to remain in AdA for an additional 15 to 50 minutes, usually 45 minutes, prior to washing and further use or storage. The PCL-modified cells were washed two times in sterile HBSS, irradiated at 10,000 R (10 Gy) and either used immediately (e.g., in an IVS assay) or were cryopreserved at -70°C until further use.

The application of hydrostatic pressure was performed in a manner similar to that previously described (Richert et al., 1986, *Cancer Immunol. Immunother.*, 22:119). Cells were dispersed in PBS or HBSS at a concentration of 10^8 cells/mL in a capped Eppendorf plastic tube (1.5 mL, Netheler and Hinz GmbH, Hamburg, FRG) and filled to the brim. A 0.5 inch (1.27 cm) 18G needle was inserted through the cap and served as a vent for pressure equalization. Both the needle and the tube were filled with PBS and the cap was pressed down without entrapping any air bubbles. Removal of all air bubbles is important, as bubbles may cause cell disruption upon release of pressure. The tubes were then placed inside a pressure bomb of 40 mL capacity (Aminco, American Instruments Co., Md.), filled with PBS or HBSS and sealed.

Pressure was gradually applied to reach a level of about 800 to 1200 atmospheres, depending on the experiment, within 7-8 minutes and this pressure was maintained for about 15 minutes. Thereafter, the bomb was unlocked and allowed to decompress gradually to ambient pressure in about 8-15 minutes. The cells were then transferred to a 50 mL tube in 10 mL of PBS and were centrifuged at 1500 rpm for 5 minutes. The pellet was then gently resuspended in PBS to the

desired cell concentration.

It is to be understood that cells may be preincubated in dialdehyde, crosslinked with dialdehyde, and then subsequently subjected to pressure as described. The exposure of preincubated cells to dialdehyde crosslinker and to hydrostatic pressure at the same time after dialdehyde preincubation in accordance with the invention comprises an improvement and is the preferred mode of PCL-modification of the present invention.

Example 3

Detection of cell surface monoaldehyde (e.g., AmA) following preincubation of cells in dialdehyde, (e.g., 2', 3' nucleoside dialdehyde AdA)

The presence of free adenosine monoaldehyde (AmA) on PCL-modified B16 melanoma cells was confirmed as follows: about 10^6 B16-BL6 cells were preincubated in AdA for various periods of time, i.e., 0 minutes or 30 minutes at different temperatures, i.e., 4°C, 25°C (approximately room temperature, RT) or 37°C, and then were subjected to PCL treatment, 10 mM AdA and 1200 atmospheres hydrostatic pressure, as described in Example 2. The number of AmA molecules per cell was determined by FACS analysis as depicted in Table 1.

To determine the presence of AmA on the cell surface, both direct fluorescence analysis and inhibition studies were performed. B16-BL6 cells were serially passaged *in vitro* every 3 to 4 days. Cells were cultured as adherent monolayers in complete medium (CM) containing RPMI 1640, 10% heat-inactivated fetal calf serum, 0.03% L-Glutamine, antibiotics (Beit Haemek Biological Industries, Israel), and 50 μM β-mercaptoethanol (Fluka, Switzerland, cat. #63690). Cells were preincubated in 10 mM AdA for either 0 or 30 minutes at the indicated temperatures and were then subjected to PCL at 1200 atm for 15 minutes. Following this, 0.5 ml of a 2 mg/ml solution of Biotin-X-Hydrazide (6-(biotinoyl)amino)caproic acid hydrazide, Molecular Probes, USA cat. # B-1600) in PBS was added to the pellet containing 10^6 cells. The cells were incubated for 1 hr at RT and then were washed 3 times in HBSS without Ca^{2+} or Mg^{2+} ($HBSS^-$). Subsequently, 100 μl of a 5 μg/ml solution of Streptavidin-FITC (Jackson

Immunoresearch Laboratories, cat. # 016-090-084) was added to the cell pellet and the cells were incubated for 1 hr at 4°C in the dark. Cells were then washed twice with HBSS⁻ and analyzed on a fluorescence activated cell sorter (FACS).

Table 1: FACS Analysis of AmA Presentation on B16-BL6 Cells after Preincubation with AdA and PCL Modification

Treatment[a]	Mean Fluorescence Channel[c]	AmA molecules/cell[d] (#AmA + cells)
Untreated[b]	1.3	900
PCL 0', 25°C	7.7(total)/29.5(22%)	6,300(26,600)
PCL 30', 4°C	38.9(total)/95.7(38%)	36,000(95,000)
PCL 30', 25°C	1,002(total)/2,385(40%)	1.2×10^6(3×10^6)
PCL 30', 37°C	871.4(total)/1791(47%)	1×10^6 (2.2×10^6)

[a] Cells were treated as described in this example. Briefly, B16-B6 cells were preincubated in 10 mM AdA for either 0 or 30 minutes at the indicated temperatures and were then subjected to PCL at 1200 atm for 15 minutes. Following this, cell surface aldehydes were fluorescence-labeled as described in this examples and FACS analysis was performed.

[b] Untreated refers to cells that were not preincubated with AdA and were not subjected to PCL treatment. During the time of these treatments, untreated cells were suspended in HBSS⁻ and maintained at RT.

[c] FACS data were collected on a FACSort cytometer. In each sample, 5000 cells were counted and all data were collected under identical conditions. The Mean Fluorescence Channel (MFC) represents the mean peak fluorescence of the sample after adjustment for nonspecific labeling. The term total indicates that the MFC was calculated taking into account 100% of the events (i.e., cells). A percent other than 100% indicates that there was more than 1 peak on the FACS analysis, and only that percent of events was taken into account in the MFC calculation.

[d] The number of AmA molecules/cell was calculated using a standard quantification kit composed of fluorescent beads loaded with a known number of fluorescein molecules (Flow Cytometry Standards Corp., San Juan, PR, USA cat.

826). A calibration plot was calculated using QuickCal (Flow Cytometry Standards Corp., San Juan, PR, USA) and the fluorescent values of unknown samples were then calculated from this calibration curve.

The results in Table 1 indicate that a 30 minute preincubation of the B16 melanoma cells with AdA prior to subjecting the cells to PCL modification resulted in an extensive amount of AmA per cell which reached maximal levels at 25°C. Other experiments have shown that the total number of AdA molecules bound per cell is on the order of 10^{10} and that the number of AmA molecules on the cell surface represents approximately 1/10,000 of bound AdA molecules, i.e., on the order of 10^6 molecules of AmA per 10^{10} molecules of total bound AdA. The effect of derivatization of the cell surface with AmA following AdA preincubation must be taken into account in the overall increased immunogenicity of surface-modulated tumor cells in experiments evaluating tumor size after immunization of animals with preincubated and PCL-modified tumor cell immunogens (see Example 8).

Example 4

Determination of the rate of membrane-bound adenosine monoaldehyde (AmA) formation and the effect of aldehyde reducing compounds

The experiment as described in Example 3 was repeated in order to determine the rate of membrane-bound AmA formation and to determine the effect of agents (i.e., inhibitors) which are known to reduce aldehydes. The results are shown in Fig. 4.

B16-BL6 cells were serially passaged *in vitro* every 3 to 4 days. Cells were cultured as adherent monolayers in complete medium (CM) containing RPMI 1640, 10% heat-inactivated fetal calf serum, 0.03% L-Glutamine, antibiotics (Beit Haemek Biological Industries, Israel) and 50 μM β-mercaptoethanol (Fluka, Switzerland, cat. # 63690). Cells were preincubated in 10 mM AdA for various periods of time (i.e., 0, 10, 20, and 60 minutes) at 4°C and then were subjected to PCL at 1,200 atm for 15 minutes. The last two columns in Fig. 4 depict the AmA levels when the PCL-treated cells were further treated with agents that reduce aldehydes. Specifically, cells were treated either with 20 mM hydroxylamine chlorhydrate (HA) (BDH, UK cat #10129), (20 + HA), or with 20 mM sodium

borhydride (SBH) (20 + SB) for 30 minutes at RT in PBS. HA and SBH are aldehyde reducing compounds which convert aldehydes to alcohols. Subsequently, 0.5 ml of a 2 mg/ml solution of Biotin-X-Hydrazide (6-((biotinoyl)amino)caproic acid hydrazide, Molecular Probes, USA cat # B-1600) in PBS was added to all of the cell pellets which contained 10^6 cells. The cells were incubated for 1 hour at RT and then were washed three times with $HBSS^-$. Following this, 100 μl of a 5 μg/ml solution of Streptavidin-FITC (Jackson Immunoresearch Laboratories, cat # 016-090-084) was added to the cell pellets and the cells were incubated for 1 hr at 4°C in the dark. Cells were then washed twice with $HBSS^-$ and analyzed on the FACS. This experiment was carried out under different conditions from the experiment depicted in Table 1, in order to reach saturating conditions of AmA.

Under the conditions of this experiment, it was determined that both aldehyde reducing compounds, HA or SBA, significantly, but not completely, reduced the amount of AmA on the cell surface. The experiment demonstrates that saturating levels of membrane-bound AmA were attained in 60 minutes and that the bound aldehyde was reduced to alcohol by added aldehyde reducing compounds.

Example 5

Effect of dialdehyde preincubation on PCL-induced immunogenicity

The effect of preincubating cells in dialdehyde AdA prior to PCL modification was assessed using B16-B6 cells as sensitizing cells and PBMC as responder cells in an *in vitro* sensitization assay as described in Example 10.

Briefly, human PBMC were prepared as described by diluting about 30 mL of heparinized blood 1:2 in sterile HBSS and separating the cell populations using Ficoll-Paque cell density gradient centrifugation (1.077 g/cm^3, Pharmacia). PBMC were harvested from the interphase of the gradient, washed two times in HBSS and stored at 4°C for up to 12 hours until use. IVS cultures were established essentially as described (see Example 10). Briefly, 2 x 10^5 freshly prepared PBMC served as responder cells in the culture and were incubated at 37°C for 5 days with 1 x 10^5 to 4 x 10^5 sensitizing B16-BL6 tumor cells (i.e., dialdehyde-preincubated, PCL-modified cells or unmodified cells (B16-UNM) as control) in 200 μL of culture medium in a 96-well microtiter plate (Nunc).

Preincubation of tumor cells in AdA dialdehyde was as described in Example 2 and was carried out for either 0 or 30 minutes. On the fifth day of culture, 1 μCi/10 μL of [³H]-thymidine was added to each well for an additional 4 hours, followed by cell harvesting using an automated cell harvester (Filtermate 196, Packard). The amount of radioactivity which reflected the amount of PBMC cell proliferation in response to the stimulating cells was measured as cpm in a liquid scintillation analyzer (Packard).

As shown in Fig. 5, a significant proliferation response resulted in those cultures in which the stimulating B16-B6 cells had been preincubated in 10 mM AdA for 30 minutes prior to subjecting the cells to PCL modification which included treatment with 10 mM AdA crosslinker and 1200 atm hydrostatic pressure (i.e., B16-PCL-30'). Virtually no proliferation was observed using unmodified responder B16-B6 cells (B16-UNM) or using responder cells that had not been preincubated in AdA prior to PCL treatment (i.e., B16-PCL-0').

Example 6

Determination of immunological responses in the presence of cell membrane-bound adenosine monoaldehyde (AmA) *in vivo*

The *in vivo* immunological effects of the presence of cell membrane-bound AmA on PCL-treated cells used as immunogens was examined in recipient animals, as shown in Fig. 6, in which the parameter of tumor growth was determined following immunization of animals with preincubated and PCL-modified tumor cells and challenge with viable tumor cells thereafter.

B16-BL6 cells were serially passaged *in vitro* every 3 to 4 days. Cells were cultured as adherent monolayers in complete medium (CM) containing RPMI 1640, 10% heat-inactivated fetal calf serum, 0.03% L-Glutamine, antibiotics (Beit Haemek Biological Industries, Israel) and 50 μM β-mercaptoethanol (Fluka, Switzerland, cat #63690). Cells were preincubated in 10 mM AdA for either 0 or 30 minutes and were subjected to PCL in which the AdA concentration was 10 mM and the hydrostatic pressure was 1200 atm for 15 minutes. The preincubated and PCL-modified cells were then washed two times in $HBSS^-$ and were used as immunogens to inoculate two month old C57BL/6J female mice. Two inoculations

were given s.c., 1 week apart, with 2 x 10' irradiated cells (10,000 R) per inoculation. One week after the second inoculation, the animals were challenged with 10' viable B16 cells and the tumor diameter was scored as a function of time on day 12, day 15, and day 19. Each group contained 6 mice.

As shown in Fig. 6, the results demonstrated that immunization of animals with an immunogen which had been prepared by a 30 minute preincubation with AdA prior to PCL-modification appeared to augment the immunogenicity of the PCL vaccine with respect to the size of the tumor (i.e., smaller tumor size). The PCL-vaccine prepared without preincubation of the cells with AdA prior to PCL modification was not immunogenic. The legend in Fig. 6 is as follows: HBSS w/o represents cells in HBSS without magnesium and calcium ("HBSS="); UT represents B16 immunogen cells that were neither preincubated in AdA nor PCL-treated, "untreated"; 0', PCL represents B16 immunogen cells that were not preincubated in AdA but were PCL-treated as described; 30', PCL represents B16 immunogen cells that were preincubated in AdA for 30 minutes and were PCL-treated as described.

Example 7

Dialdehydes as adjuvants/immunopotentiators of the cell response to proliferating agents

Experiments were performed to assess the effect of the dialdehyde AdA to enhance or augment the proliferative response of PBMC to the anti-CD3 antibody OKT3 in *in vitro* culture via its immunopotentiating or adjuvanting abilities. PBMC were assayed as described in Example 11 to determine their proliferative response to OKT3. Cells were incubated either with or without OKT3; either with or without various concentrations of AdA (0.01 to 50 μM); either with or without 700 μM DHA (dioxoheptanoic acid, a Schiff base forming ketone); or with combinations of these compounds as shown in Fig. 7. The extent of proliferation by PBMC was measured by standard methods and as described. It can be seen from the results shown in Fig. 7 that the presence of AdA in concentrations as low as 0.01 μM in combination with OKT3 causes a significantly higher level of proliferation of PBMC to OKT3 compared with OKT3 alone

(columns 6-10 versus column 3). Interestingly, when OKT3 was present in the culture with AdA at a concentration of 50 μM, a significant reduction in the proliferative response of PBMC to OKT3 was observed, thus suggesting that higher concentrations of AdA are not effective as adjuvants and do not have immunostimulating function. The dialdehyde AdA as immunopotentiator in culture with PBMC enhanced the proliferation of PBMC to OKT3 as well as or better than the presence of 700 μM of DHA in the cultures with OKT3 (compare column 4 with columns 6-10). These results demonstrate that AdA causes immunopotentiation or costimulation of the proliferation response of PBMC to OKT3 at an approximately ten thousand fold lower concentration than does the compound DHA (i.e., 0.01 μM versus 700 μM, respectively).

Example 8

Preparation of human tumor cells from resected human tumors

Freshly resected tumors (usually 1-3 x 1-3 x 1-2 cm in size) were transferred in the cold (approximately 4°C) within 1 hour from the operating room (OR). Tumors were transferred to a 100 mm petri dish, rinsed 3-5 times in 10 mL cold PBS, and then transferred to a second petri dish where necrotic and fatty tissue were removed. The tumor tissue was cut into small pieces (approximately 1-5 mm^2 each), transferred to a 500 mL plastic flask containing: 100 mL of RPMI medium, 50 mg collagenase (Sigma), 1500 units DNase type IV, 5 mg Hyaluronidase type V, 0.01 M Hepes (Biological Industries, Israel), 0.03% L-glutamine (Biological Industries, Israel), Pen 5000 units/Strep 5 mg (Biological Industries, Israel), 1:20 dilution of Fungizone (Biomycin-2, Biological Industries, Israel), and 25 mg gentamycin (Biological Industries, Israel). After stirring the tumor in the enzymatic solution for 2-3 hours (depending on the tumor size), the tumor cell suspension was transferred to a 50 mL plastic centrifuge tube through a 120 micron nylon mesh, and washed one time in phosphate buffered saline (PBS) or Hank's Balanced Salt Solution (HBSS). The cell pellet was then resuspended in 35 mL PBS and layered gently on top of 15 mL of Ficoll gradient (1.077 g/cm^3). After centrifuging for 20 minutes at 450 x g at room temperature, cells in the interphase were collected, washed twice in PBS or HBSS by centrifuging at room

temperature at 400 x g for 10 minutes, counted for viability using trypan blue exclusion, and diluted or concentrated to obtain the desired cell number.

One of skill in the art will appreciate that in patients bearing a tumor, the tumor may be wholly or partially excised by biopsy or surgery employing techniques and practices known to the skilled practitioner. Appropriate precautions are taken for safety and sterility. Individual tumor cells may be dissociated into single cell suspensions or dispersions using conventional enzymatic, chemical, or mechanical means.

It is also noted that the tumor cell suspension obtained as described herein from freshly resected tumor tissue and used in accordance with the invention represents a heterogeneous population of cells of which about 20% to about 70% is comprised of tumor cells and about 30% to about 80% is comprised of a mixture of mononuclear cell types from peripheral blood, including accessory cells (e.g., macrophages, monocytes, or antigen-presenting cells) and lymphocytes, that reside within the tumor tissue (for example, see Table 5). When such freshly obtained tumor cell preparations are used for PCL modification, the accessory cells resident in the tumor are also capable of being modified. It is envisioned that such PCL modification of the non-tumor accessory cells or antigen-presenting cells and lymphocytes, which are part of the tumor preparation, may affect or enhance the presentation of antigenic molecules, either alone or in combination with MHC proteins and the like, when these PCL-modified cells are used in association with PCL-modified tumor cells in an immunogenic preparation, thereby allowing a better presentation of immunogenic structures by these cells to the lymphocytes, particularly, the T lymphocyte subsets, of the immune system.

Example 9

Preparation of cells from solid tumor or ascites

A subcutaneous tumor was carefully excised, minced with scissors into fragments 1-2 mm in size, and stirred in a triple-enzyme mixture of hyaluronidase, deoxyribonuclease and collagenase (Sigma Chemical Co., St. Louis, Mo.) for 30-60 min in HBSS= (Ca^{2+}-and Mg^{2+}-free) as described by Lafreniere, R. and S.A. Rosenberg. 1986. "A novel approach to the generation and

identification of experimental hepatic metastases in a murine model", *JNCI*, 76:309. The suspension was then collected and passed through 100-μm nylon mesh, washed three times in HBSS, and resuspended at the appropriate concentration. The cells were either frozen in aliquots at -70°C for 24 h and then transferred to liquid nitrogen and stored for later use, or serially passaged in vitro every 3-4 days. Cells were cultured as adherent monolayers in tissue-culture flasks (Falcon 3024), seeded at approximately $3x10^5$ cells/75-cm^2 flask in 20 mL complete medium containing RPMI-1640, heat-inactivated fetal calf serum (10% v/v), penicillin (100 U/mL), streptomycin (100 μg/mL), 0.03% fresh L-glutamine, 0.1 mM non-essential amino acids, 0.1 μM sodium pyruvate, 50 μg gentamicin/mL, 0.5 μg solubilized amphotericin B (Sigma Chemical Co., St. Louis, Mo.) and 50 μM 2-mercaptoethanol. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2, and passaged every 3-4 days. Cells were harvested from confluent B16-BL6 monolayers by draining the medium, washed twice with HBSS and then were overlaid with 1-2 mL trypsin/versene (Gibco) for 2-3 min while the bottom of the flask was tapped occasionally. Digestion was stopped by the addition of 10 mL complete medium. Detached cells were washed twice in HBSS and resuspended at the desired concentration.

Example 10

In vitro sensitization (IVS) assay

The proliferative response of effector cells, i.e., PBMCs or lymphocytes, in the presence of tumor cells was carried out as follows: For human experiments using human PBMCs, 2 x 10^5 viable PBM cells in 0.1 mL of conditioned medium were co-cultured with irradiated tumor cells (10,000 R) in the wells of a 96-well microtiter plate (final concentration of tumor cells in 0.1 mL was 5 x 10^4 to 5 x 10^3). Tumor cells were either PCL-treated or PCL-untreated. Tumor cells were prepared by cutting the tumors into small pieces (approximately 1 mm), followed by enzymatic digestion for 2-3 hours, and then separating viable cells on a cell separation gradient (450 x g for 20 minutes at room temperature), or such as is described in Examples 2 and 4. PBMC were isolated from 20 mL of blood taken from the patient, followed by dilution (1:1) in sterile PBS or HBSS

and separation on a cell separation gradient as described for tumor cells. PCL-modification was carried out by exposing 5 x 10^6 to 1 x 10^7 cells (either tumor cells or PBMCs) to 1200 atmospheres of hydrostatic pressure in the presence of 40 mM AdA. Thereafter, cells were irradiated at 10,000 R. The endpoint of the IVS assay was the measurement of cell proliferation which correlates directly with the extent of stimulation. Cell proliferation was measured by adding [^{3}H]-thymidine for the final 6 hours of incubation of a 5 day IVS assay. Cells were then harvested using a cell harvester (Packard) and radioreactivity retained on the filters was counted using a beta counter (Packard).

For mouse experiments using B16-BL6 cells, samples containing 2 x 10^5 viable cells, i.e., splenocytes, were co-cultured with 1 x 10^5 irradiated (50 Gy) PCL-treated or untreated B16-BL6 cells in a 96-well flat-bottomed microplate (Nunc Denmark), for 48 h at 37°C in a humidity-controlled incubator under a 5% CO_2 atmosphere. The culture medium consisted of RPMI-1640 medium plus 10% heat-inactivated fetal calf serum supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). Cultures of effector cells and "stimulator" tumor cells were pulsed with [methyl-^{3}H]thymidine (Amersham) after about 42 h of culture, and after 6 h, the cells were harvested and the incorporated radioactivity was measured by conventional methods.

It is pointed out that *in vitro* sensitization assays may also be described as mixed lymphocyte culture (MLC) assays. In MLC, T cells respond to foreign histocompatibility antigens on unrelated lymphocytes or monocytes. The test may be performed as either a "one way" or a "two way" assay. In a one way MLC assay, the stimulating cells are treated with either irradiation (approximately 1500-2000 R) or with mitomycin to prevent DNA synthesis without killing the cells. The magnitude of the response is the result of DNA synthesis measured in the non-irradiated or non-mitomycin treated cells. In a two way MLC, DNA synthesis of both stimulating and responding cells represents the net response of both sets of cells. The individual contributions of each cell population cannot be discerned. Controls include co-culture of syngeneic irradiated and nonirradiated pairs (to determine baseline DNA synthesis) and co-culture of allogeneic irradiated

pairs (to determine adequate inactivation by irradiation).

More specifically, for a one way MLC, responder peripheral blood lymphocytes are mixed 1:1 with irradiated stimulator cells and incubated at 37°C in a humidified atmosphere with 5% CO_2. After 5 days, the culture is pulsed with [^{3}H]-thymidine to label the nucleic acid in the responder cells. After 18 hours, the cells are harvested and counted for internalized radioactivity. For example, if the MHC or HLA antigens of the stimulator cells differ from those of the responder cells, the responder cells undergo blastogenesis, synthesize DNA, and proliferate; increased sample radioactivity is the result. If there are no MHC or HLA difference, the cells remain quiescent and no increase in radioactivity is measured at the end of the assay.

Example 11

Anti-CD3 and PHA stimulation of PBMCs

In the wells of a 96-well microtiter plate, $2x10^5$ cells were incubated in 0.2 mL tissue culture medium, e.g., RPMI (Gibco), supplemented to contain 20 μL of either (a) anti-CD3 monoclonal antibody or PHA in a 96-well microplate. Anti-CD3 antibody was obtained in culture supernatant prepared from hybridoma cell line number 454 (Dr. J. Lawrence, Cornell Medical Center, N.Y.). Other sources of anti-human CD3 antibody can be used. For example, OKT3, a hybridoma cell line which produces anti-CD3 monoclonal antibody directed against human peripheral T cells, is available through the ATCC, e.g., ATCC CRL 8001. Another anti-human CD3 monoclonal antibody-producing hybridoma cell line is also available through the ATCC, ATCC HB231. For these assays, both anti-CD3 monoclonal antibody and PHA were diluted 1:20 in culture medium.

Example 12

Delayed-type hypersensitivity (DTH) assay

For non-human animals, the DTH response was measured by skin reaction in the ear as described by Vadas et al., 1975. Int. Arch. Allergy Appl. Immunol. 49:670. For immunization, unmodified or modified tumor cells were

irradiated (10,000 rad) and then injected i.p. into C57BL/6J female mice at a dose of 10^7 viable cells in 1 mL of PBS/mouse (cell counts were determined before irradiation). After 8 days, immunization was repeated as above with a fresh batch of unmodified or modified tumor cells.

A sample of 10^5 unmodified and irradiated tumor cells (an empirically determined optimal dose) in 10 μL PBS was injected intradermally 8 days later in the right ear (0.5 in., 1.27 cm, 30G needle, Becton Dickinson, N.J.). The left ear (control ear) was injected with 10 μL PBS. After 10 hours, the mice were injected i.p. with 0.1 mL of a 1.0 mM 5-fluoro-2′ deoxyuridine (FdUrd, Sigma) solution. 30 minutes later, the mice were injected i.v. with 2 μCi of 5-^{125}I-labeled 2′-deoxyuridine (^{125}IdUrdR, sp. act. 5 Ci/ng, Amersham, UK) in the lateral tail vein.

Mice were sacrificed after 24 h from the time of challenge with tumor cells. The ears were then cut out carefully at the rims and the amount of radioactivity determined in a gamma counter (Gammamatic, Kontron). The results were expressed as the ratio of radioactivity in the right ear to that in the left ear (R/L ^{125}IdUrd index). Five mice were included in each group. Control groups included unprimed mice, as well as those primed with unmodified tumor cells.

In human patients, to determine the immunogenicity of autologous or allogeneic tumor cells PCL-modified in accordance with the invention and used as immunogens, patients were evaluated at baseline using the DTH skin test. The patients were given two separate subcutaneous or intradermal injections, side by side, of unmodified (i.e., no PCL treatment) and of PCL-modified tumor cells (1 x 10^5 to 1 x 10^6 cells/injection). The cells were irradiated by a dose of 10,000 rad. so that they lost their ability to proliferate, and yet still maintained their immunogenic potential. In particular, 25 patients received immunogens comprising autologous tumor cells modified with crosslinking and pressure treatment in accordance with the invention.

The development of the skin test reaction or immune reaction in humans (i.e., DTH) was scored at 24, 36, 48, and 72 hours after injection of the cells. 24-48 hours were frequently optimal and normal for observing the peak of a

reaction. An immune reaction elicited by the injected cells was evidenced by a swollen, red area which appeared in the skin at the inoculation site. The degree of a patient's skin reaction was determined by the diameter of the redness (erythema) and the degree of swelling (induration), as described by Scornick et al. 1981. *Cancer Immunother. Immunol.*, 11:93.

DTH skin reactions were scored as follows: "-", "+", "++", or "+++", where "-" indicates no reaction and "+++" indicates maximal redness and swelling reactions. Alternatively, DTH responses can be scored numerically, such that a score of "3" indicates a very strong response (and corresponds to the above-described "+++" response); a score of "2" indicates a moderate response, corresponding to "++"; and a score of "0-1" indicates a minimal or poor DTH response, corresponding to "+", above.

Example 13

Preparation of plasma membranes from cells and isolation of cytosolic and membrane-associated or shed protein and protein complexes from cells

Plasma membranes were prepared from cells, e.g., tumor cells, essentially as described by Maeda, T. et al. 1983. *Biochim. Biophys. Acta.*, 731:115. Briefly, unmodified or PCL-modified tumor cells were homogenized in cold Hank's Balanced Salt Solution (HBSS) containing EDTA, PMSF, and DNAse using a polytron homogenizer with three cycles of homogenization, at 5 seconds per cycle. The crude homogenate was first centrifuged at low speed (i.e., about 800 rpm) to remove debris and nuclei. The supernatant was collected and layered on top of a 41% sucrose solution and centrifuged at 91,000 x g for 60 minutes. The interface band was carefully aspirated using a pasteur pipet and was centrifuged at 100,000 x g for 90 minutes. The pellet was then resuspended in a small volume of HBSS. Protein content was determined by Lowry's Folin-Ciocalteau assay (Lowry, O.H. et al. 1951. *J. Biol. Chem.*, 193:265).

Methods to prepare cytosolic and membrane proteins from cells. Cells are centrifuged in cell medium and the pelleted cells are washed once in PBS. For tumor isolates, tumor cells are dispersed in cell medium by mincing with a scalpel prior to centrifuging. The washed cells are resuspended in a hypotonic buffer A

(Buffer A: 10 mM KCl, 10 mM HEPES, pH 8.0, 1 mM EDTA/EGTA, protease inhibitors and phosphatase inhibitors) at a final cell density of about 10 million per mL in buffer A for about 15 to 30 minutes on ice. Thereafter, 63 μL of 10% NP-40 or Triton-X® 100 is added per mL of cell suspension (i.e., about 0.6% nonionic detergent final concentration). The cell and detergent mixture is vortexed for about 15-30 seconds, and centrifuged in an microcentrifuge (Eppendorf) for about 1 minute. The resulting cell pellet contains cell debris (i.e., connective tissue) and nuclei. The resulting cell supernatant contains cytosolic proteins and solubilized plasma membrane proteins.

To isolate the cytosolic cell protein fraction only, cells are resuspended in buffer A, quick frozen in liquid nitrogen, thawed, and centrifuged for about 30 minutes in a microcentrifuge (Eppendorf). The resulting supernatant contains predominantly cytosolic proteins. To isolate the plasma membrane protein fraction, the cell pellet resulting from the above-described 30 minute centrifugation is extracted on ice for about 30 minutes in buffer A containing 0.5% NP-40 or Triton-X® 100 and is then centrifuged for 5 minutes in a microcentrifuge (Eppendorf). The soluble fraction contains predominantly plasma membrane proteins and residual cytosol.

In another method, cells are treated with crosslinker and pressure in accordance with the invention (i.e., about 10 to 20 mM 2′, 3′ nucleoside or nucleotide dialdehyde, at the same time that the cells are exposed to about 800 to 1400 atm hydrostatic pressure; most preferably 10 mM crosslinker and 1200 atm pressure). The PCL-treated cells are then subjected to hydrostatic pressure of greater than or equal to about 1600 atm and the cells are centrifuged to pellet cell debris. The resulting cell supernatant is applied to a G100 or G250 column, whereby the fractions of crosslinked proteins are isolated. Such a high pressure method allows the collapse of the cell membrane structure and the corresponding release and isolation of soluble proteins or protein complexes, some or all of which have their hydrophilic portions in association with membrane lipids.

Example 14

In vivo immunization and survival studies of animals vaccinated with an

immunogenic preparation comprising modified B16/Bl6 melanoma cells and non-classical adjuvant to enhance the immune response

Experiments are performed to test the survivability of C57BL/6 mice immunized prophylactically with immunogenic preparations comprising B16/BL6 tumor cells that are preincubated in 10 mM dialdehyde AdA and then PCL-modified by exposing the tumor cells to AdA crosslinker at 10 mM at the time that the cells are also subjected to hydrostatic pressure of 1200 atmospheres. The immunogenic preparations also contain either GM-CSF or HGH as a non-classical adjuvant. Unmodified or untreated cells are used as controls as described.

BL6/BL6 melanoma cells are a very invasive variant of the B16 cell line (Hart 1979, *Am. J. Pathology*, 97:587) and are obtained from B16/BL6 tumors that are serially passaged in syngeneic C57BL/6 mice by subcutaneous (s.c.) inoculation of 2 - 5 x 10^6 cells.

In the *in vivo* experiments described in this example, the viability of C57BL mice challenged with 1 x 10^5 viable non-PCL-treated B16/BL6 tumor cells is tested following immunizations with the immunogenic preparations comprising B16/BL6 cells, either unmodified or PCL-modified in accordance with the invention. The cells used for vaccination are of the same kind as the cells used to challenge the mice.

The immunization protocol comprises two vaccinations, i.e., injections of an immunogenic preparation comprising about 20 x 10^6 PCL-modified B16/BL6 tumor cells formulated with either HGH or GM-CSF at concentrations of 1 μg/mL to 100 μg/mL per injection, administered one week apart. The dose concentrations used for each adjuvant are typically 1, 5, 10, 20, 50, and 100 μg/mL. One week after the last vaccination, animals are challenged with tumor cells. Control immunogens contain unmodified cells (e.g., in medium such as Hank's Balance Salt Solution, HBSS) with and without the presence of adjuvant, as well as PCL-modified cells without adjuvant. In general, 6-10 mice are immunized to test the controls and each immunogenic preparation containing PCL-modified cells and the various doses of adjuvant.

After the immunizations with the immunogen preparations, the

immunized animals are challenged by injection with viable B16/BL6 tumor cells and the ability of immunized animals to survive the challenge is assessed over a period of about 45 days or longer post-challenge.

The following parameters are tested for each group of animals described in this example: survival rate and the tumor number and mean tumor diameter (mm^2) measured using standard callipers in three orthogonal directions and presented by the mean value.

Example 15

DTH response in a melanoma patient treated with allogeneic PCL-treated melanoma cells and GM-CSF as non-classical adjuvant

DTH analyses were performed in a human patient to assess the DTH response to PCL-immunogens administered in combination with non-classical adjuvants, such as GM-CSF. A melanoma patient was immunized with an immunogen comprising PCL-modified allogeneic melanoma cells in conjunction with non-classical adjuvant, i.e., GM-CSF. It is to be understood that autologous melanoma cells may also be used. Prior to the immunization protocol using PCL-modified immunogen and adjuvant, the patient was screened or pretested in a baseline DTH assay to select the optimal adjuvant dose of GM-CSF to use during the immunization protocol. In the DTH baseline screening assay, 1 x 10^7 PCL-treated allogeneic melanoma cells were injected subcutaneously (SC) at sites 2 inches apart in the patient's forearm. PCL-treated cells were injected alone (0.5 cc) or mixed with 20 μg (low dose) or 100 μg (high dose) of GM-CSF (Leukine, available from Immunex Corp.) in 0.1 cc. Cells for immunization were PCL modified as described using 10 mM AdA and 1200 atm pressure. The GM-CSF injections were repeated using the respective low and high doses at 24 and 48 hours at the DTH immunization sites. The development of the DTH response was scored at 24 and 48 hours after immunization by measuring the diameter or size of the area of induration at the immunization site using callipers and as known by those in the art. For example, if the DTH response area was essentially circular, a single diameter was measured and reported; if the response area was non-circular or irregular in shape, the area was measured in two dimensions (at two independent

positions) and the mean of the measurements was determined. Based on the patient's response to the DTH baseline assay, a given dose of GM-CSF (i.e., 100 μg) was selected for use in the PCL immunization protocol.

The immunization protocol was generally carried out about 48 hours following the baseline DTH assay and comprised a course of three subcutaneous injections, most preferably at sites near a draining lymph node. For the initial injection (day 0), the patient was immunized SC (e.g., in the forearm or in the thigh) with 1 x 10^7 PCL-treated cells, together with 100 μg of GM-CSF (0.6 cc total). In general, the immunizing dose of PCL-modified cells as immunogen was on the order of about, or greater than, ten times the number of cells used in the DTH screening assay (e.g., 1 x 10^7 cells versus 1 x 10^6 cells in the DTH screen). The second injection at 24 hours comprised GM-CSF adjuvant alone (100 μg) at the same site and the third injection at 48 hours comprised adjuvant alone (100 μg). As described herein, IVS assays were performed at several intervals (e.g., at four and six weeks) following the immunization protocol to assess the patient's immune response status and potency level. At the end of the protocol, DTH assays were again performed as described to evaluate the patient's immune response to both PCL modified and unmodified cells.

The use of GM-CSF as adjuvant revealed a clear and significant augmentation of the patient's DTH immune response in a dose-dependent manner, as evidenced by the size of the area of the induration (i.e., the DTH response area) at the immunization site after injections with 10^6 PCL-treated melanoma cells and either 20 or 100 μg of GM-CSF administered as adjuvant as described. The results are presented in Table 2:

TABLE 2

Immunogen	Adjuvant GM-CSF	Size of DTH Response Area (Induration) at Immunization Site: Induration 24 hours after immunization (cm^2)	Induration 48 hours after immunization (cm^2)
$1x10^5$ PCL-treated allogeneic melanoma cells	–	0.2 x 0.5	–
$1x10^5$ PCL-treated allogeneic melanoma cells	20 μg	2.1 x 2.5	2.0 x 2.1
$1x10^5$ PCL-treated allogeneic melanoma cells	100 μg	3.0 x 3.5	2.5 x 2.9

In addition, DTH response evaluations such as those described in this example are performed using the dialdehyde and antigen compositions of the invention, wherein melanoma cells as immunogen are replaced with a protein or peptide antigen, or portion thereof, such as a tumor associated antigen (TAA) derived from melanoma cells as described hereinabove. Dialdehyde AdA and melanoma antigen are incubated in a 10:1 or 20:1 ratio for 30 minutes at room temperature, the resulting composition is admixed with carrier or excipient, as necessary or desired, and used to immunize a patient to test the resulting DTH response. AdA is used at 10 to 20 mM. The composition may contain a non-classical adjuvant component; alternatively, the adjuvant, e.g., GM-CSF or hGH, may be co-administered.

Example 16

Dialdehyde and protein compositions

Compositions comprising dialdehyde and protein antigen are prepared for use as immunogens for enhancing, stimulating or potentiating the immune response to antigen. The antigen used in the composition is baculovirus-produced polyoma virus middle T protein antigen and the dialdehyde is AdA.

Other antigens are also suitable for use as described herein. 10 mM AdA is admixed and reacted with antigen in HBSS, and the mixture is allowed to incubate for 30 minutes at room temperature. As a general guide, 10 to 20 mM dialdehyde incubated with antigen for 30 minutes at room temperature provides a dialdehyde and antigen composition in accordance with the invention for use as immunogen. In addition, the ratio of dialdehyde to antigen, e.g., protein or peptide, may be on the order of 10:1 to 20:1. The dialdehyde may be mixed with the antigen to produce the dialdehyde and antigen preparation immediately prior to use, or the two components may be mixed and cryopreserved for subsequent use, depending upon the stability of the particular antigen. The AdA and antigen immunogenic preparation is injected into mice.

Mice are injected s.c. either with antigen alone, with the composition comprising antigen plus the dialdehyde 2′,3′-adenosine dialdehyde (AdA), or with antigen plus 100 μg of a suitable adjuvant or carrier. Additional injections of the aforementioned test compounds are provided subsequently for up to four days at the same injection site. After a week to 10 days inguinal (regional) lymph nodes are removed and the lymph node cells are restimulated with antigen alone. Antigen specific proliferation of lymphocytes is measured after a 5 day IVS assay. Tests for humoral immunity are performed by administering antigen and test compounds in the same way. After 1 to 2 weeks, blood is sampled by venepuncture and serum antibody is assayed by enzyme linked immunosorbant assay (ELISA). Such experiments are expected to show that the dialdehyde and antigen-containing compositions of the invention are potent enhancers of immune responses in mice.

The ability of the dialdehyde and antigen-containing compositions of the invention to induce cell-mediated immunity and the activation of cytotoxic T cells, which is important to fighting infection and tumors is assessed. Mice a are immunized subcutaneously with composition of AdA (10 μg) plus keyhole limpet hemocyanin (50 μg) as antigen and are boosted with the same composition 3 to 5 weeks later. After a further 6 to 8 weeks, and a final boost with the same composition, spleens are harvested. Harvested spleen cells may be further

stimulated *in vitro* with antigen for 5 days prior to performing the cytotoxic assay using conventional procedures. Target cells (P815 cells, a mastocytoma) are pulsed with antigen and with an irrelevant antigen (e.g., the NP peptide of influenza virus) and labeled with ^{51}Cr. Spleen and target cells are incubated for 4 to 6 hours and killing is measured by the release of ^{51}Cr expressed as a percentage of the total released from detergent lysed cells. Spontaneous release is also measured. Percent killing is measured as a function of the effector (spleen) to target (labeled P815) cell ratio. Cytotoxic T cells are expected to be activated in response to immunization with the dialdehyde and antigen compositions of the invention.

Similar experiments are performed using the dialdehyde and antigen-containing compositions injected in the presence of nonconventional adjuvant such as hGH and GM-CSF. Alternatively, these adjuvants are mixed with the composition such that both the antigen and the adjuvant cytokine are reacted with the two monoaldehyde groups of the dialdehyde component of the composition. Comparison is made with the immune response results obtained using immunogens with and without nonconventional adjuvant.

Example 17

Augmentation of the T cell response against EBV-associated B-cell lymphoma using dendritic cells modified by pressure and crosslinking: Enhancement of TH1 cytokines and precursor frequency of anti-tumor CTLs

In this example, PCL treatment to create cell-based immunogens/vaccines for *ex vivo* generation of tumor-specific CTLs as a proposed regime for the treatment of lymphoma, e.g., EBV-associated lymphoma, was examined.

Generation of Cell Lines

EBV-specific T-cell lines:

EBV-transformed B lymphocyte cell lines (BLCL) were established as described in A. Toubert et al., 1984, "Identification of several functional subgroups of HLA-B27 by restriction of the activity of antiviral T killer lymphocytes", *Immunogenetics*, 20:513. B95.8 cells, which are a marmoset tumor cell line used to propagate EBV (ATCC Accession No. CRL 1612), were grown to

confluence in RPMI supplemented to contain 20% FBS. Cells were centrifuged and resuspended at 1.2 x 10^6 cells/ml. Cells were incubated at 37°C, 5% CO_2 in a humidified incubator for three days, after which time supernatant was harvested to use for infection. 20 x 10^6 peripheral blood mononuclear cells (PBMC) were incubated overnight in 2 ml EBV supernatant with 20 μl cyclosporin. The following day, maintenance medium (i.e., 10 ml AIM V + 1:1000 (v/v) β-mercaptoethanol + 1:1000 (v/v) gentamicin) was added and immortalized BLCLs were maintained in culture.

Dendritic Cell Lines:

Dendritic cell (DC) lines were initiated as follows: 400 x 10^6 PBMCs were plated in T225 flasks in maintenance medium (described above) and were incubated for 2 hours in a 37°C, 5% CO_2 humidified incubator. Two-hour supernatant was removed and non-adherent cells were frozen and stored for future use. Additional maintenance medium was added containing GM-CSF (800 U/ml) and IL-4 (500 U/ml). Cells were cultured for 8 days, and then were harvested using cell dissociation medium. Cells were frozen for future use or used immediately.

T Cell Lines:

T-cell lines were initiated as follows: 4 x 10^6 BLCL were irradiated (6000 RADS) and combined with 4 x 10^6 DC for each T cell group. Cells were incubated for 2 hours in low volume maintenance medium. 80 x 10^6 cells from 2-hour supernatant (as generated above) were added to each flask with BLCL and DC (4 x 10^6 cells). Maintenance medium was added to a total volume of ~15 ml and the flasks were incubated for 3 days. Thereafter, nonadherent cells were transferred to new flasks. As the maintenance medium turned a light orange color, cell lines were split and additional medium was added.

Pressure and Cross-Linking (PCL) Modification

For PCL-modification, cells (30 x 10^6) were suspended in 2 ml AdA (10 mM) crosslinker solution. This was considered the start time for a 30 minute incubation of the cells in crosslinker solution. The tips of 2 ml sterile transfer pipettes were cut off with sterile scissors. The cell suspension was then aliquoted

among transfer pipettes using Pasteur pipettes. The tops of the transfer pipettes were folded over and sealed with metal clips using a sealing instrument. Sealed transfer pipettes were then checked for bubbles. If bubbles were present, the cells were transferred to new transfer pipettes by the above-described process.

Properly sealed pipettes containing cell suspension were then transferred to the pressure chamber of the PCL device. After thirty minutes, the cells were gradually brought up to 1,200 atm hydrostatic pressure, sustained at this pressure for 15 minutes, and then slowly brought back to 1 atm pressure. Following PCL, transfer pipettes containing the cell suspension were placed in 15 ml centrifuge tubes and centrifuged at 1200 rpm for 7 minutes. Supernatant was then removed very carefully from the transfer pipettes using Pasteur pipettes. Cells were suspended in sterile PBS and were transferred to new sterile 15 ml centrifuge tubes. Cells were then washed three times more in PBS. After the last wash, the cells were resuspended in maintenance medium until further use.

Proliferation assay

Autologous and allogeneic BLCLs and K562 cells were counted and irradiated (6000 RADS). Cells were serially diluted in maintenance medium and plated in 96-well U-bottom plates in concentrations indicated for the individual experiment. Identical plates containing BLCLs and K562 cells were plated for each T cell group. T cells were counted and plated over BLCLs at 50,000 cells/well. Additional control plates containing BLCLs alone and T cells alone were established. The total volume of each well was 200 μl maintenance medium. Plates were incubated for 72 hours at 37°C, 5% CO_2 in a humidified incubator. If cytokine assays were to be performed, 100 to 150 μl of supernatant was removed and stored at 4°C. Thereafter, approximately 1 μCi ^{3}H-Thymidine was added per well in a small volume of maintenance medium. Cells were incubated in radiolabel overnight and were harvested using the Tomtec Cell Harvester (Wallac, Gaithersburg, MD). ^{3}H-Thymidine incorporation was determined using the Wallac Microbeta plate reader (Wallac, Gaithersburg, MD). Net counts were determined by subtracting background counts obtained from plates housing BLCLs and T cells alone. A stimulation index was determined by dividing net counts by counts of T

cells alone.

Cytokine assay

ELISA kits (Endogen and R&D Systems) were used to measure cytokine release from cells in the cell cultures. Supernatants from all T cell groups and BLCL concentrations were plated in duplicate on pre-coated ELISA plates. Cytokine standards were also plated in duplicate. Biotinylated antibody was plated over supernatant. Plates were covered and incubated at room temperature for 2 hours and then were washed three times using wash buffer as provided by the supplier. Streptavidin-HRP concentrate was diluted in dilution buffer and 100 μl were added to each well. The plates were covered and incubated at room temperature for 30 minutes. The plates were again washed three times using wash buffer. Premixed TMB substrate solution was added to each well. Plates were developed in the dark for ~30 minutes and the reaction was stopped by adding stop solution (100 μl) to each well. Absorbance was read on a plate reader set to 450 - 550 nm. A standard curve was generated using absorbance values obtained from cytokine standards. The amounts of cytokines produced from experimental T cell groups incubated with various concentrations of stimulators were then calculated using standard curves that had been generated.

Limiting dilution analysis

To prepare a feeder cell layer, PBMC from a healthy donor buffy coat were isolated, washed, and irradiated (6000 RADS). Cells were again washed and were resuspended at 2 x 10^6 cells/ml maintenance medium + 50 U/ml rhIL-2. Cells were plated in one 96-well U-bottom plate for each T cell group. To prepare effector cells, T cells from each experimental group were counted and resuspended at 1 x 10^6 cells/ml in maintenance medium. Cells were serially diluted 10-fold to produce a series of 6 dilutions (10^5, 10^4, 10^3, 10^2, 10, and 1 cell(s) per well).
T cells were plated over the feeder cell layer. Anti-CD3 (200 ng/ml) was added to all of the wells. The total well volume was 200 μl/well in maintenance medium. The plates were incubated in a 37°C, 5% CO_2 humidified incubator for 14 days. On days 3 and 10, 50 μl/well cell-free supernatant was removed and replaced with 50 μl/well fresh maintenance medium containing 100 U/ml rhIL-2. On day 7, 50

μl/well cell-free supernatant was removed and replaced with fresh maintenance medium containing 1 x 10^5 PBMC irradiated feeder cells (6000 RADS) and 100 U/ml rhIL-2. On day 14, the cells in each well were resuspended and distributed uniformly into one 96-well plate for each target cell group. Additional maintenance medium was added to bring the volume in each well to 100 μl/well.

Cells were assayed on day 14. 100 μl/well target cells were plated over T cells to be assayed. A control cell plate was set up containing cells for obtaining spontaneous and maximum release values for each target. The plates were incubated for 5-7 hours at 37°C, 5% CO_2 in a humidified incubator. 100 μl/well were removed and combined with an equal volume of scintillation fluid. The plates were read using a Wallac Microbeta plate reader, and the frequency of CTL precursors was determined.

As a result of this experiment in which EBV-associated B cell lymphoma lines were subjected to PCL modification and co-cultured with autologous PBMC, primary T cell lines were shown to proliferate and secrete high levels of γIFN, RANTES, and MIP 1-α upon stimulation with autologous, HLA-compatible B-cell lymphoma tumor cells, but not after stimulation with allogeneic B-cell lymphoma or unrelated tumor cells (Fig. 10 and Figs. 16A-D). In addition, low levels of the counter-regulatory cytokine, IL-10, were secreted after antigenic stimulation (Fig. 11 and Fig. 14). Thus, PCL-modification induced the creation of T cell lines exhibiting a more vigorous proliferative response and an enhanced secretion of TH1 cytokines, compared with T cells generated following stimulation with unmodified tumor cells.

Dendritic cells pulsed with cell extract prepared from a pool of various allogeneic EBV-associated B-cell lymphoma were used as a cellular cancer immunogen/vaccine to generate tumor-specific T cell lines. DC-pulsed with tumor antigen were shown to trigger a strong T cell activation against the B cell lymphoma, as measured by proliferative response and cytokine secretion (Fig. 12 and Fig. 13). For DC pulsing, whole cell extract (WCE) was prepared from tumor cells by repeated freeze-thaw. The concentrations or levels of the proteins in the WCE was measured using a commercially available kit for this purpose (Pierce).

Approximately 1 x 10' DC were incubated with 100 μg of WCE for about 5 to 16 hours. Thereafter, the cells were washed and used in IVS assays as stimulators for T cells. The ratio of responders to stimulators (R:S) was about 10-20:1. PCL modification of DC-pulsed with tumor extract added a dramatic increase to their immunogenic potential compared with unmodified DC-pulsed with tumor antigen.

Primary T cell lines derived from PBMC and comprising both CD4+ and CD8+ T cell subsets were stimulated with B-cell lymphoma antigens which were either presented endogenously by intact tumor cells or presented by DC following antigen processing of exogenous tumor cell extract. Co-culture of T cells with either anti-CD4 MoAb or anti-CD8 MoAb resulted in an impartial inhibition of the T cell proliferative response (Fig. 15). Thus, PCL-modified cells (tumor or DC-pulsed with tumor antigen) induced the generation of tumor-specific T cells of both the CD4+ and the CD8+ phenotypes.

To determine the precursor frequency of tumor-specific CTLs elicited by the various forms of cellular immunogens created and employed in this example, the above-described limiting dilution analysis (LDA) was performed. PCL-modification of intact tumor cells conferred an increased immunogenicity summed up to a magnitude of 2 logs. To lyse 50% of tumor (target) cells in a microcytotoxicity assay, 10,000 T cells generated following stimulation with unmodified tumor cell vaccine were required. Following stimulation with PCL-modified, intact tumor cells, merely 100 T cells were required to lyse 50% of the target cells.

The presentation of tumor antigens to T cells by professional APC (DC-pulsed with tumor cell antigen) was shown to be highly effective. Treatment of loaded DC to PCL modification further enhanced the potency of APC presentation to T cells. Specifically, the PCL treatment reduced the number of T cells required to lyse 50% of target cells to approximately 2 cells per well compared with 100 cells per well achieved with T cells generated following stimulation with unmodified APC (Fig. 8). Thus, tumor cell presentation by APC combined with PCL treatment created a powerful cell immunogen which exhibited the capacity to elicit high affinity tumor-specific CTLs from peripheral blood

lymphocytes. It is likely that such an immunogen can be used to break tolerance to cancer cell antigens in order to provoke a highly specific and protective T-cell response, to abrogate metastatic dissemination, thereby halting disease progression.

Accordingly, a comparison of the killing capacity of T cells generated by the various cell-based immunogens using LDA revealed that PCL treatment of intact autologous tumor cells elicited CTL precursor frequency that was 100 fold higher than that elicited by unmodified tumor cells. Following tumor cell stimulation with PCL-modified antigen-loaded DC, CTL precursor frequency increased an additional 100. Thus, PCL-modification was demonstrated to augment the presentation of tumor antigens to T cells by professional APC (DC), thereby creating a potent anti-tumor immunogen with exceptional capacity to elicit highly efficient CTLs to eradicate cancers, tumors and infected cells.The contents of all patents, patent applications, published articles, books, and abstracts cited herein are hereby incorporated by reference in their entirety to more fully describe the state of the art to which the invention pertains.

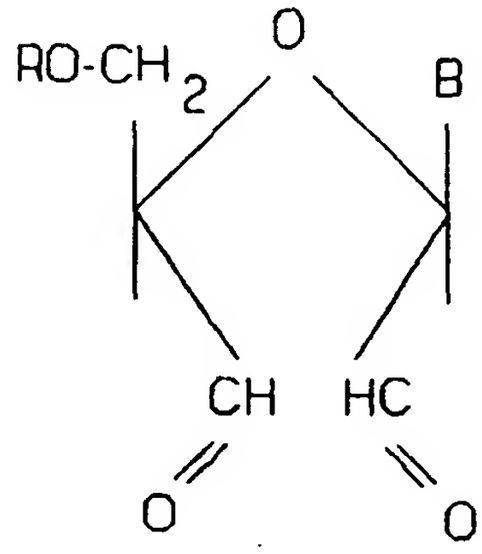
The contents of all patents, patent applications, published articles, books, reference manuals and abstracts cited herein are hereby incorporated by reference in their entirety to more fully describe the state of the art to which the invention pertains.

As various changes can be made in the above-described subject matter without departing from the scope and spirit of the invention, it is intended that all subject matter contained in the above description, shown in the accompanying drawings, or defined in the appended claims will be interpreted as descriptive and illustrative, and not in a limiting sense. Many modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced in a broader sense than that as specifically described and/or exemplified.

WHAT IS CLAIMED IS:

1. A composition for enhancing or augmenting an immune response against an antigen associated with a cancer, a tumor or an infection caused by a pathogen, comprising a dialdehyde component and the antigen component.

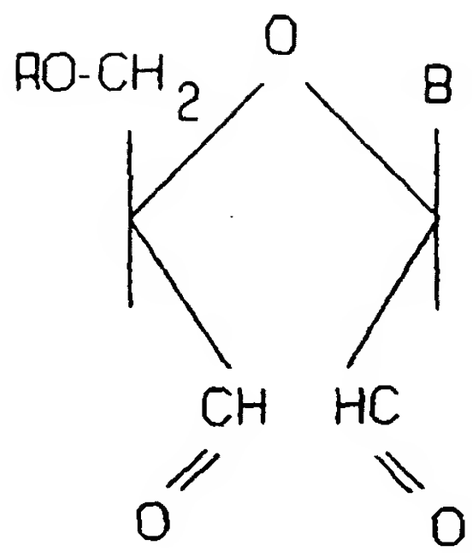
2. The composition according to claim 1, wherein the dialdehyde is malonic dialdehyde, glutaric dialdehyde, or a 2′, 3′-nucleoside or nucleotide dialdehyde having the formula:



wherein, R is H, or a mono-, di- or tri-phosphate group, and
B is a nucleotide base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil.

3. An immunogenic composition for enhancing or augmenting an immune response against cancer cells, tumor cells, or cells infected with a pathogen, comprising antigen-presenting cells exposed to 2′, 3′ nucleotide or nucleoside dialdehyde crosslinker and to hydrostatic pressure, wherein said crosslinker and pressure-treated antigen-presenting cells are antigen activated by i) co-culturing with intact cancer, tumor or infected cells, or membranes thereof, or ii) pulsing with at least one antigen associated with said cancer, tumor or infected cells, thereby resulting in antigen processing and expression by the crosslinker and pressure-treated antigen-presenting cells.

4. The composition according to claim 3, wherein the 2′, 3′-nucleoside or nucleotide dialdehyde has the formula:



wherein, R is H, or a mono-, di- or tri-phosphate group, and

B is a nucleotide base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil.

5. The composition according to any of claims 1 to 4, wherein the dialdehyde is 2′, 3′-adenosine dialdehyde (AdA) or 2′, 3′-adenosine monophosphate dialdehyde (AMPdA).

6. The composition according to claim 1 or claim 3, wherein the antigen is a cancer, tumor or infected cell, or a noncellular protein, peptide, or carbohydrate derived from the cancer, tumor or infected cell.

7. The composition according to claim 1 or claim 3, wherein the antigen is a cell or a noncellular protein, peptide or carbohydrate antigen derived from viruses, bacteria, protozoa, yeast, algae, or fungi.

8. The composition according to claims 3 to 5, wherein the antigen presenting cells are activated by exposure to antigens associated with a cancer, a tumor, or a pathogen.

9. The composition according to claim 8, wherein the cancer- or tumor- or pathogen-associated antigen is selected from the group consisting of proteins, a peptides, carbohydrates and membrane fragments.

10. The composition according to claim 8 or 9, wherein the antigen- presenting cells are activated by exposure to antigens associated with a pathogen selected from the group consisting of viruses, bacteria, protozoa, yeast, algae, and fungi.

11. The composition according to any of claims 1-5, further

comprising a biological response molecule or an adjuvant.

12. The composition according to claim 11, wherein the biological response molecule or adjuvant is coupled to the antigen-presenting cells.

13. The composition according to claim 11, wherein the biological response molecule or adjuvant is coupled to a component of the composition via a monoaldehyde moiety.

14. The composition according to claim 11, wherein the biological response molecule is a lymphokine, a cytokine, a growth factor, or an immunostimulatory molecule.

15. The composition according to claim 14, wherein the lymphokine, cytokine, growth factor, or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, G-CSF, hGH, TNFα, IFNγ, RANTES, B7-1, B7-2, IL-1α, IL-1ß, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

16. The composition according to claim 15, wherein the biological response modifier is granulocyte-macrophage colony stimulating factor or human growth hormone.

17. The composition according to claim 15, wherein the dialdehyde component and the antigen component are mixed prior to administration.

18. The composition according to claim 1 or 2, wherein the dialdehyde component and the antigen component are injected independently at a site of administration to interact at the injection site in situ.

19. The composition according to claim 1 or 2, wherein said composition is injected in conjunction with an adjuvant or immunostimulatory molecule.

20. The composition according to claim 19, wherein the adjuvant is a cytokine, a lymphokine, or a chemokine.

21. The composition according to claim 20, wherein the adjuvant

is human growth hormone or granulocyte-macrophage colony stimulating factor.

22. The composition according to claim 1 or 2, further prepared with a therapeutic compound.

23. The composition according to claim 3 to 5, wherein the antigen presenting cells are selected from the group consisting of dendritic cells, macrophages, astroctyes, fibroblasts, fibrocytes, Langerhans cells and endothelial cells.

24. The composition according to claim 23, wherein the antigen presenting cells are genetically engineered to express exogenous genes for antigen presentation.

25. The composition according to claim 23, wherein the antigen presenting cells are immortalized or transformed.

26. The composition according to claim 3-5 or 11-16, further comprising cytotoxic T cells, wherein said T cells have been proliferated by co-culturing with intact cancer, tumor or infected cells, or membranes thereof, and further wherein the cancer, tumor or infected cells used for co-culturing with T cells are derived from the same source as the cancer, tumor or infected cells used to activate the antigen-presenting cells.

27. The composition according to claims 3-5, wherein the cancer, tumor or infected cells used for co-culturing are subjected to crosslinker and hydrostatic pressure treatment prior to co-culturing with the antigen-presenting cells.

28. The composition according to claim 27, wherein the cancer, tumor or infected cells used for co-culturing are subjected to crosslinker and hydrostatic pressure treatment prior to co-culturing with the antigen-presenting cells or the T cells.

29. The composition according to claim 26, wherein the source of cytotoxic T cells is human blood peripheral mononuclear cells.

30. The composition according to claim 26, wherein said T cells are autologous or allogeneic to a mammal.

31. The composition according to claim 3-5, wherein the source

of antigen-presenting cells is human blood peripheral mononuclear cells.

32. The composition according to claim 3-5, wherein the exposure to dialdehyde crosslinker and hydrostatic pressure comprises preincubating the antigen-presenting cells in the dialdehyde in an amount and for a time effective to crosslink cell membrane components and to generate monoaldehyde groups on the cell surface prior to subjecting the preincubated cells to a crosslinking effective amount of crosslinker and hydrostatic pressure of between 500 to 1400 atmospheres so as to modify the plasma membranes of the cells and preserve the formation of the monoaldehydes.

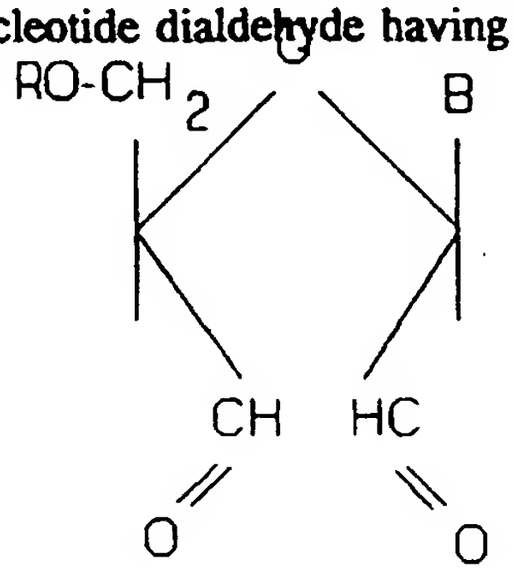
33. The composition according to claim 32, wherein the amount of dialdehyde effective for preincubation is in the range of 8 mM to 30 mM.

34. The composition according to claim 33, wherein the amount of dialdehyde effective for preincubation is 10 mM.

35. The composition according to claim 32, wherein the preincubation is from about 20 minutes to about 60 minutes.

36. The composition according to claim 32, wherein the preincubation is for 30 minutes.

37. The composition according to claim 32, wherein the dialdehyde for preincubation and for crosslinking the antigen-presenting cells is a 2′, 3′-nucleoside or nucleotide dialdehyde having the formula:



wherein, R is H, or a mono-, di- or tri-phosphate group, and

B is a nucleotide base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil.

38. The composition according to claim 37, wherein the

dialdehyde is 2′, 3′-adenosine dialdehyde (AdA) or 2′, 3′-adenosine monophosphate dialdehyde (AMPdA).

39. The composition according to any of claims 1-38, wherein the cancer or tumor is selected from the group consisting of pancreatic tumors, ovarian tumors, cervical tumors, melanomas, sarcomas, breast cancers, gastric cancers, colon cancers, lung cancers, cancers of the head and neck, brain tumors, laryngeal cancers, rectal cancers, small lung carcinomas, non-small lung carcinomas, renal cancers, epitheliomas, bladder cancers, prostate cancers, lymphomas, non-Hodgkins lymphoma, and leukemias.

40. The composition according to claim 1 or claim 3, wherein the pathogen is selected from the group consisting of viruses, bacteria, parasites, protozoa, yeast, algae, and fungi.

41. A method for reducing or alleviating a tumor or cancer in a mammal comprising administering to the mammal in need of treatment a therapeutically effective amount of the composition according to any of claims 1 to 22.

42. The method according to claim 41, wherein the tumor or cancer is selected from the group consisting of pancreatic tumors, ovarian tumors, cervical tumors, melanomas, sarcomas, breast cancers, gastric cancers, colon cancers, lung cancers, cancers of the head and neck, brain tumors, laryngeal cancers, rectal cancers, small lung carcinomas, non-small lung carcinomas, renal cancers, epitheliomas, bladder cancers, prostate cancers, lymphomas, non-Hodgkins lymphoma, and leukemias.

43. A method for reducing or alleviating a DNA or RNA virus infection in a mammal comprising administering to the mammal in need of treatment a therapeutically effective amount of the composition according to claims 1 to 22.

44. The method according to claim 43, wherein the virus is selected from the group consisting of influenza virus, human immunodeficiency virus, papilloma virus, hepatitis viruses A, B, C, D, E, F, and G, cytomegalovirus, polio virus, respiratory syncytial virus, herpes simplex virus,

Epstein Barr virus, adenovirus, rhinovirus, rabies virus, foot and mouth disease virus, equine flu, feline immunodeficiency and feline leukemia viruses.

45. A method for reducing or alleviating a bacterial infection in a mammal comprising administering to the mammal in need of treatment a therapeutically effective amount of the composition according to claims 1 to 22.

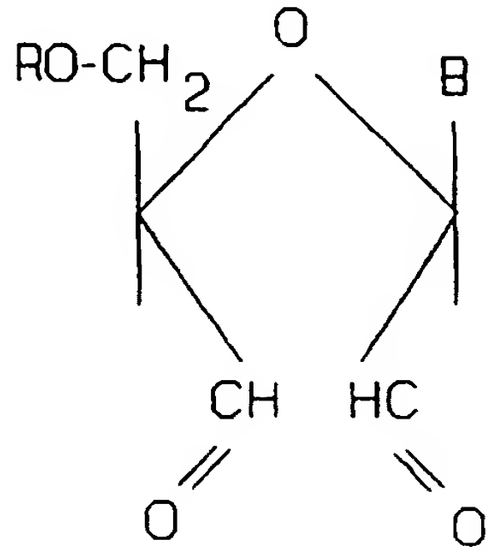
46. The method according to claim 45, wherein the bacteria are selected from the group consisting of B. pertussis, C. tetani, N. meningitidis, N. gonorrhea, S. aureus, S. pneumoniae, H. influenzae, C. diptheriae, P. aeruginosa, V. cholerae, E. coli, B. subtilis, Campylobacter jejuni and Campylobacter pylori.

47. A method for preparing a immunogenic composition for enhancing or increasing an immune response against an antigen associated with a tumor, a cancer or an infectious disease-causing pathogen, comprising:

a) mixing a dialdehyde with the antigen at a concentration and for a time effective to chemically bind the dialdehyde to the antigen by means of a first aldehyde group of the dialdehyde and to generate an unbound aldehyde group by means of a second aldehyde group of the dialdehyde, to form a dialdehyde and antigen composition; and

b) washing unbound dialdehyde from the composition.

48. The method according to claim 47, wherein the dialdehyde is malonic dialdehyde, glutaric dialdehyde, or 2′, 3′-nucleoside or nucleotide dialdehyde having the formula:



wherein, R is H, or a mono-, di- or tri-phosphate group, and B is a nucleotide base selected from the group consisting of adenine, guanine, cytosine, thymine, and

uracil.

49. The method according to claim 48, wherein the dialdehyde is 2′, 3′-adenosine dialdehyde (AdA) or 2′, 3′-adenosine monophosphate dialdehyde (AMPdA).

50. The method according to claim 47, wherein the antigen is a cell or a noncellar protein, peptide, or carbohydrate antigen selected from the group consisting of tumor cells, cancer cells, virus-infected cells, parasite-infected cells, bacteria-infected cells, yeast-infected cells, parasite cells, bacterial cells, and yeast cells.

51. The method according to claim 47, wherein the dialdehyde and the antigen are mixed for about 30 to 60 minutes at room temperature.

52. The method according to claim 47, wherein the dialdehyde is present at a concentration of about 10 mM to 20 mM.

53. The method according to claim 47, wherein the ratio of dialdehyde to antigen in the mixing step is 10:1 to 20:1.

54. The composition according to claim 1 or 2, produced by the steps comprising:

a) incubating the dialdehyde and the antigen at a concentration and for a time effective to 1) chemically bind the dialdehyde to the antigen by means of a first aldehyde group of the dialdehyde and 2) generate an unbound pendant aldehyde group by means of a second aldehyde group of the dialdehyde, to form a dialdehyde and antigen composition; and

b) washing unbound dialdehyde from the composition.

55. A method for eliciting a T cell immune response against antigens associated with cancer, tumor or infected cells, comprising:

a) exposing antigen-presenting cells to cancer, tumor or infected cell-associated antigens to achieve specific antigen presentation and activation by the antigen-presenting cells;

b) incubating the antigen-activated antigen-presenting cells in 2′, 3′ nucleotide or nucleoside dialdehyde crosslinker in an amount and for a time effective to crosslink cell membrane components and to generate

monoaldehyde groups on the cell surface;

c) treating the antigen-presenting cells of step b) with hydrostatic pressure to modify the plasma membranes of the cells and preserve the formation of the monoaldehydes, thereby producing a population of PCL-modified antigen-presenting cells; and

d) co-culturing the PCL-modified antigen-presenting cells with T cells, wherein the T cells have been activated to proliferate and secrete TH1-type cytokines by incubation with PCL-modified cancer, tumor, or infected cells expressing cancer, tumor or infected cell-associated antigens.

56. The method according to claim 55, wherein the hydrostatic pressure is about 500 to 1400 atmospheres.

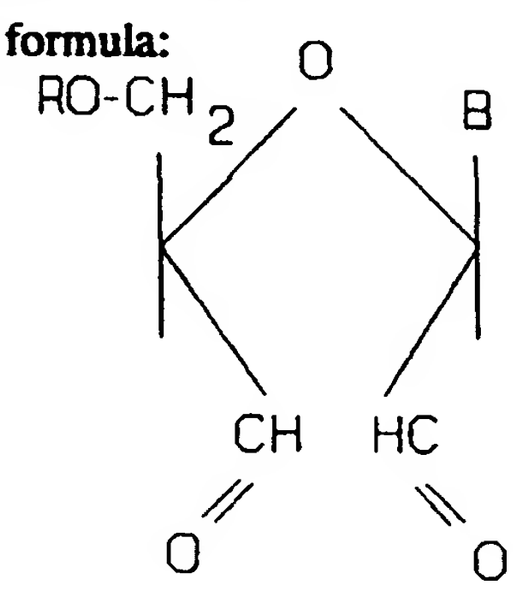
57. The method according to claim 56, wherein the hydrostatic pressure is about 800 to 1200 atmospheres.

58. The method according to claim 55, wherein in step b) the dialdehyde is present at a concentration of about 8 mM to 30 mM.

59. The method according to claim 58, wherein the dialdehyde is present at a concentration of about 10 mM.

60. The method according to claim 55, wherein the incubation step b) is from about 20 minutes to about 60 minutes.

61. The method according to claim 55, wherein the dialdehyde for preincubation and for crosslinking cells is a 2′, 3′-nucleoside or nucleotide dialdehyde having the formula:



wherein, R is H, or a mono-, di- or tri-phosphate group, and

B is a nucleotide base selected from the group consisting of adenine, guanine,

cytosine, thymine, and uracil.

62. The method according to claim 61, wherein the dialdehyde is 2′, 3′-adenosine dialdehyde (AdA) or 2′, 3′-adenosine monophosphate dialdehyde (AMPdA).

63. The method according to claim 55, wherein the antigens of step a) are cancer or tumor associated antigens.

64. The method according to claim 63, wherein the tumor or cancer-associated antigens are expressed on cells of cancers or tumors selected from the group consisting of pancreatic tumors, ovarian tumors, cervical tumors, melanomas, sarcomas, breast cancers, gastric cancers, colon cancers, lung cancers, cancers of the head and neck, brain tumors, laryngeal cancers, rectal cancers, small lung carcinomas, non-small lung carcinomas, renal cancers, epitheliomas, bladder cancers, prostate cancers, lymphomas, non-Hodgkins lymphoma, and leukemias.

65. The method according to claim 55, wherein the the antigens of step a) are RNA or DNA virus associated antigens on virus-infected cells.

66. The method according to claim 65, wherein the virus-infected cells are infected with viruses selected from the group consisting of influenza virus, human immunodeficiency virus, papilloma virus, hepatitis viruses A, B, C, D, E, F, and G, cytomegalovirus, polio virus, respiratory syncytial virus, herpes simplex virus, Epstein Barr virus, adenovirus, rhinovirus, rabies virus, foot and mouth disease virus, equine flu, feline immunodeficiency and feline leukemia viruses.

67. The method according to claim 55, wherein the antigens of step a) are associated with cells infected with bacteria.

68. The method according to claim 67, wherein the bacteria are selected from the group consisting of B. pertussis, C. tetani, N. meningitidis, N. gonorrhea, S. aureus, S. pneumoniae, H. influenzae, C. diptheriae, P. aeruginosa, V. cholerae, E. coli, B. subtilis, Campylobacter jejuni and Campylobacter pylori.

69. The method according to claim 55, wherein the antigens of step a) are associated with cells infected with yeast, protozoans, fungi, or parasites.

70. The method according to claim 55, wherein the antigen-

presenting cells of step c) are treated with dialdehyde crosslinker and hydrostatic pressure at the same time following step b).

71. The method according to claim 55, further comprising the step of coupling a biological response molecule to the PCL-treated cells of step c).

72. The method according to claim 71, wherein the biological response molecule is a lymphokine, a cytokine, a growth factor, or an immunostimulatory molecule.

73. The method according to claim 72, wherein the lymphokine, cytokine, growth factor, or immunostimulatory molecule is selected from the group consisting of ICAM-1, ICAM-2, LFA-1, LFA-3, CD72, GM-CSF, G-CSF, hGH, TNFα, IFNγ, RANTES, B7-1, B7-2, IL-1α, IL-1ß, Il-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, G-CSF, M-CSF, IFNα, CTAP III, ENA-78, GRO, I-309, PF-4, IP-10, LD-78, MGSA, MIP-1α, MIP-1B, and combinations thereof.

74. The method according to claim 73, wherein the biological response modifier is granulocyte-macrophage colony stimulating factor (GM-CSF) or human growth hormone (hGH).

75. The method according to claim 55, wherein the antigen-presenting cells are selected from the group consisting of dendritic cells, macrophages, astroctyes, fibroblasts, fibrocytes, Langerhans cells and endothelial cells.

76. The method according to claim 55, wherein the antigen-presenting cells are genetically engineered to express exogenous genes for antigen presentation.

77. The method according to claim 55, wherein the antigen-presenting cells are immortalized or transformed.

78. The composition according to claim 32-38, wherein the antigen presenting cells are treated with dialdehyde crosslinker and hydrostatic pressure at the same time following the preincubation in dialdehyde crosslinker.

79. A vaccine comprising the composition according to any of claims 1-40 or 78 in a pharmaceutically acceptable vehicle, carrier or excipient.

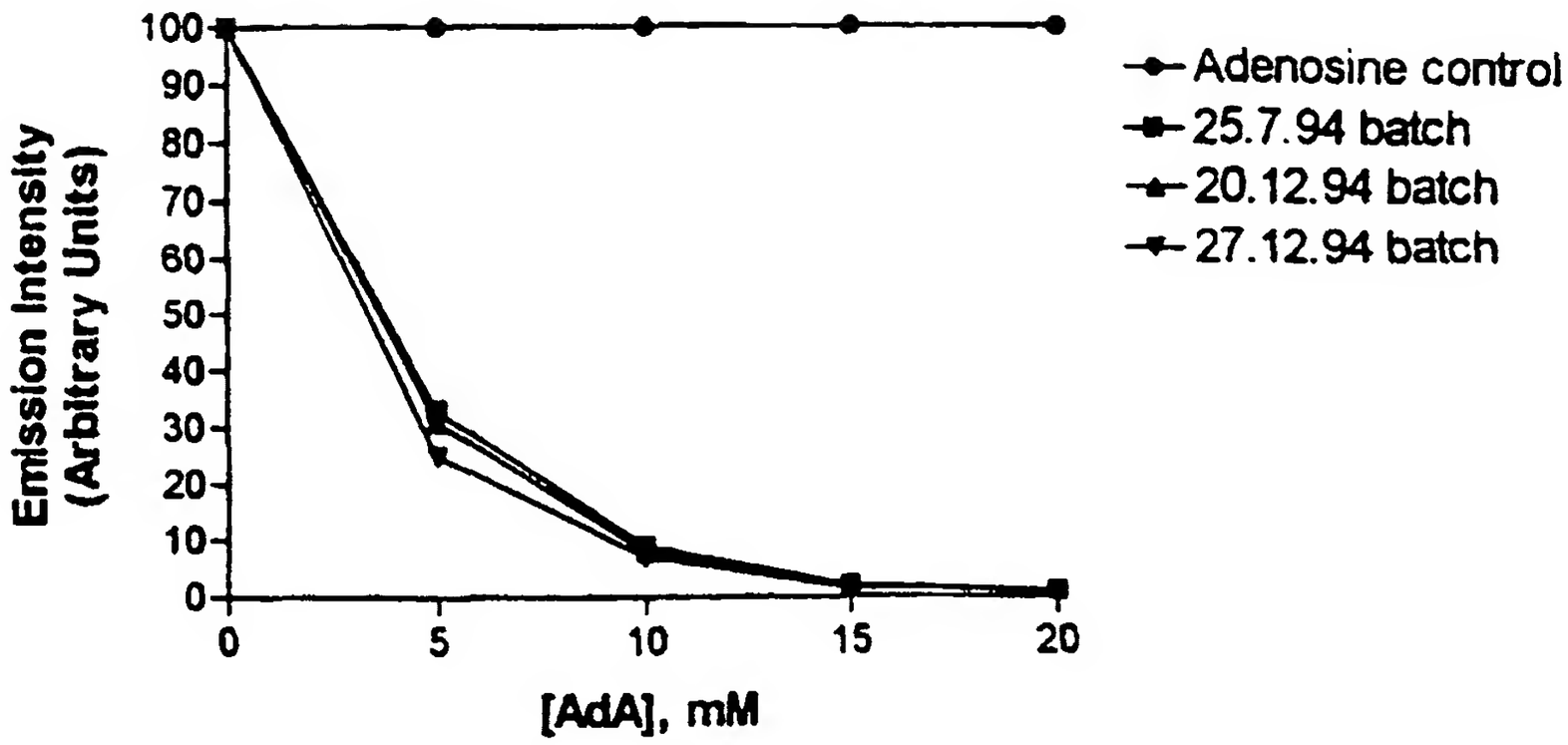

Emission Intensity (Arbitrary Units)
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
[AdA], mM
Adenosine control
25.7.94 batch
20.12.94 batch
27.12.94 batch

FIG. 1

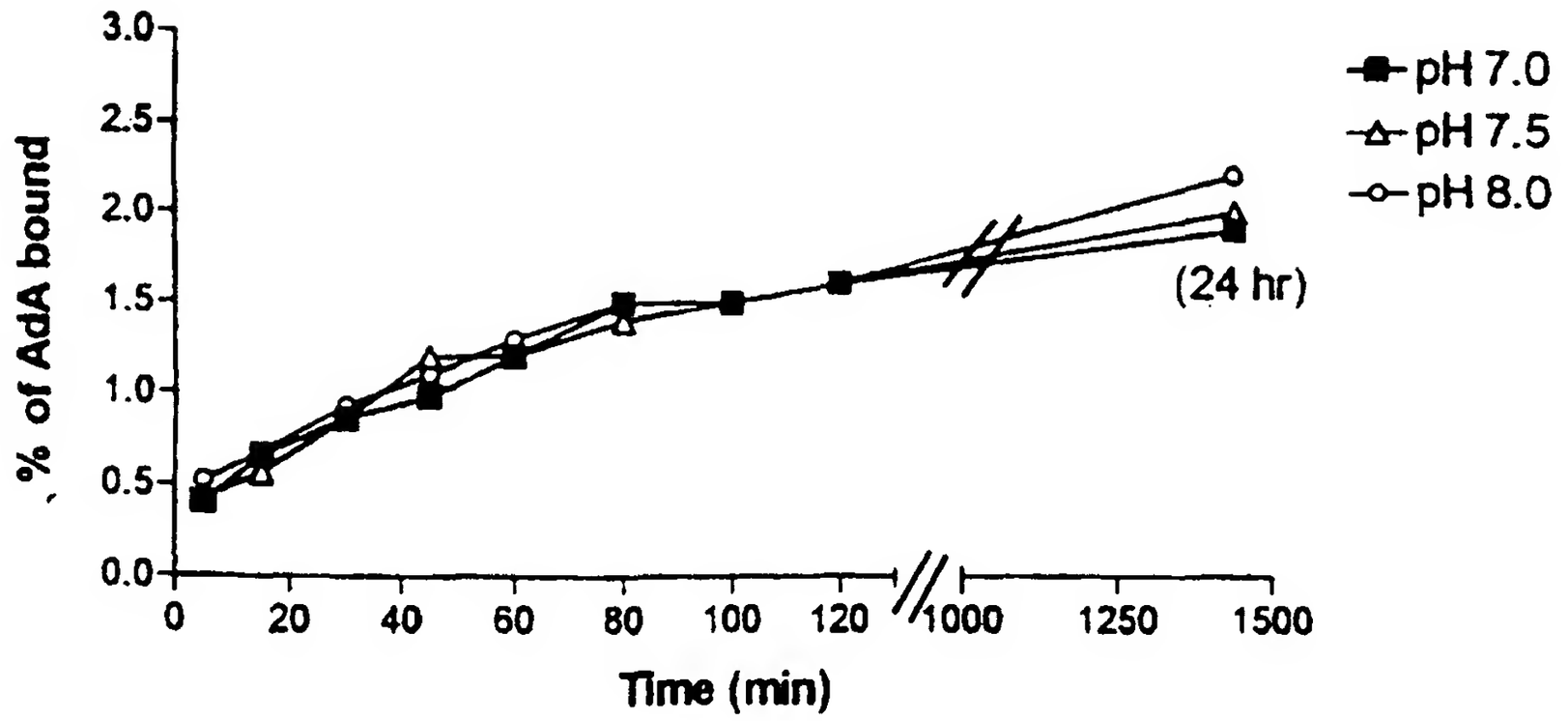

% of AdA bound
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
1000
1250
1500
Time (min)
(24 hr)
pH 7.0
pH 7.5
pH 8.0

FIG. 2

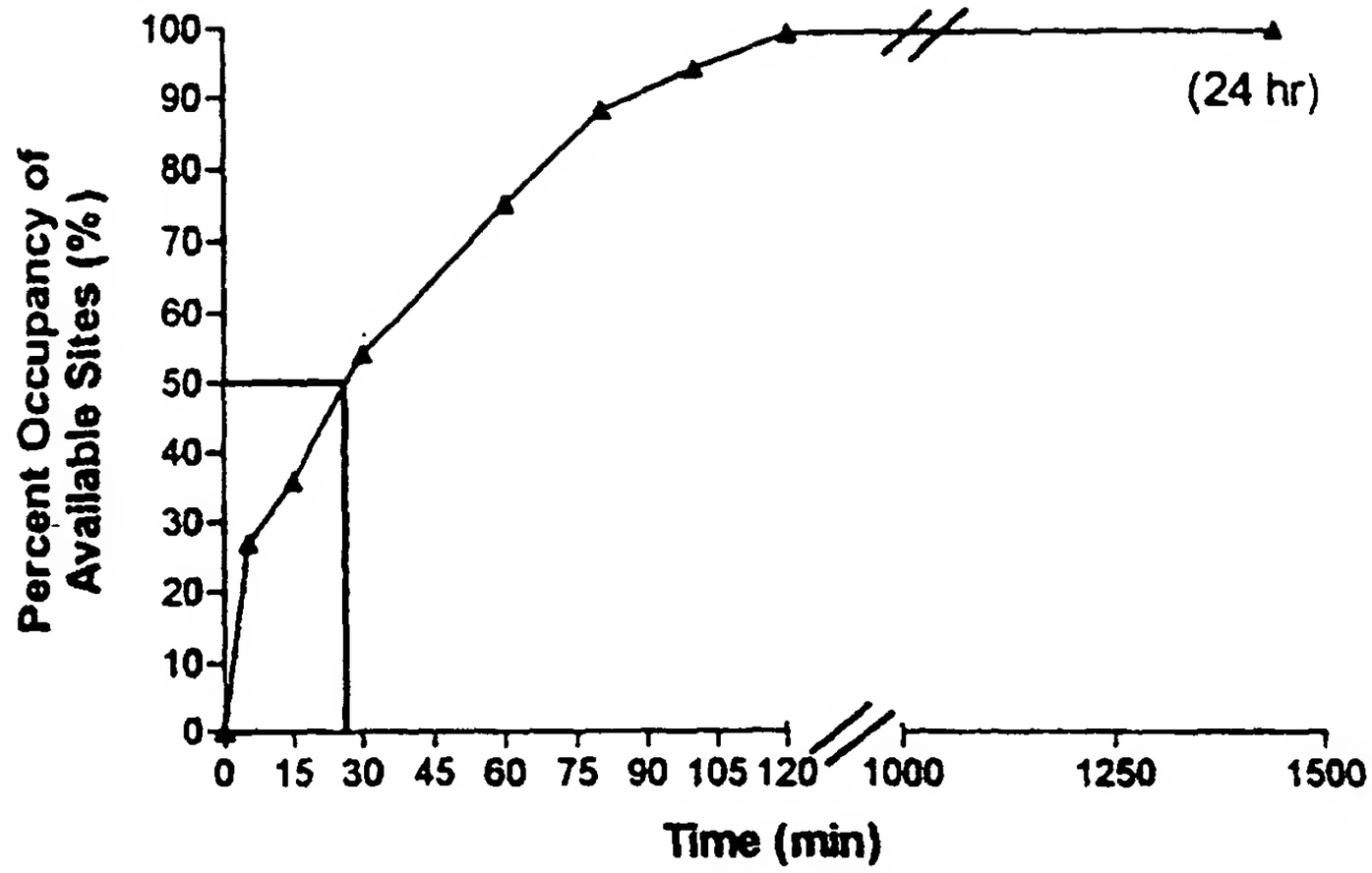

Percent Occupancy of Available Sites (%)
100
90
80
70
60
50
40
30
20
10
0
0 15 30 45 60 75 90 105 120 1000 1250 1500
Time (min)
(24 hr)

FIG. 3

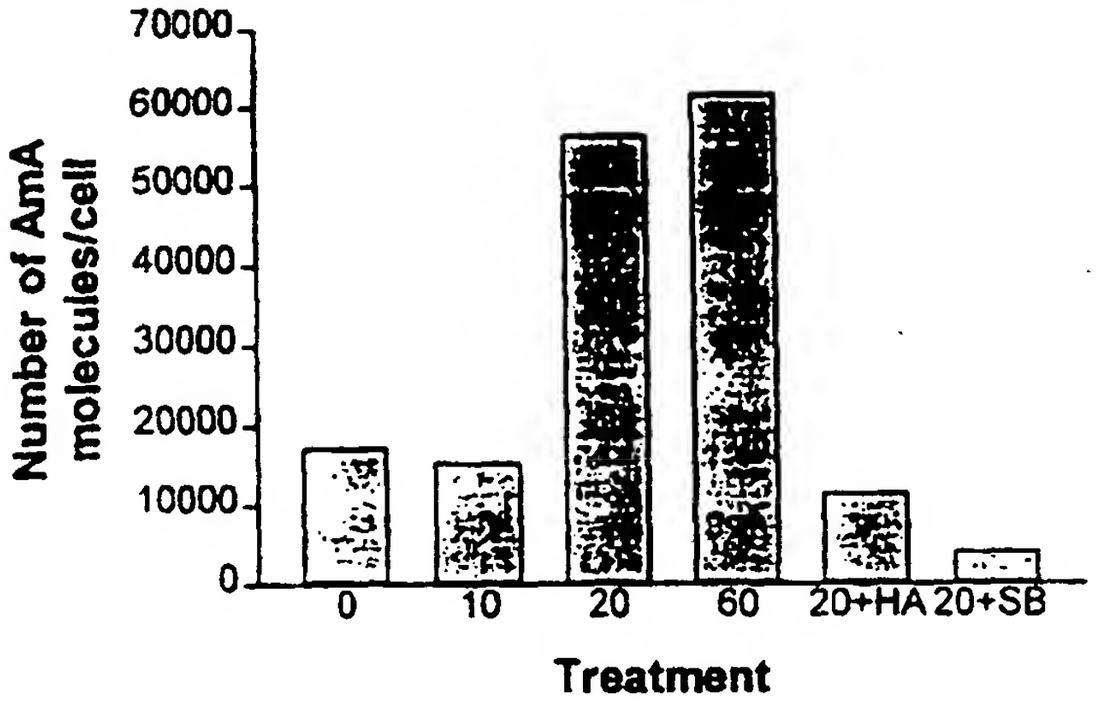

Number of AmA molecules/cell
70000
60000
50000
40000
30000
20000
10000
0
0 10 20 60 20+HA 20+SB
Treatment

FIG. 4

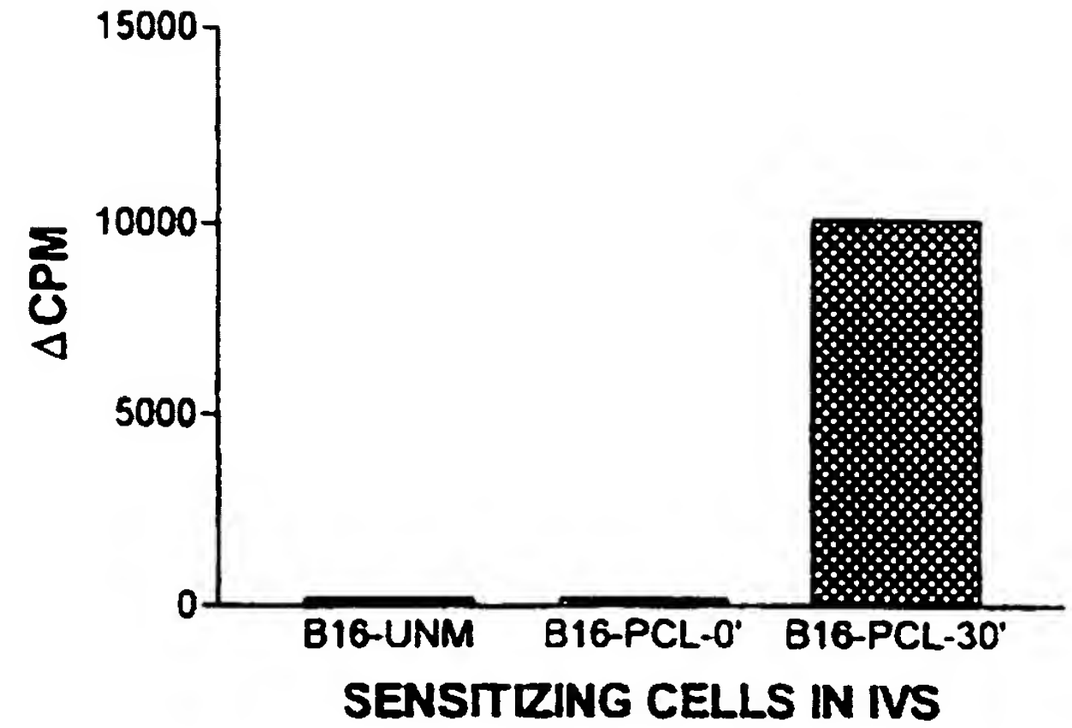

15000
10000
5000
0
ΔCPM
B16-UNM
B16-PCL-0'
B16-PCL-30'
SENSITIZING CELLS IN IVS

FIG. 5

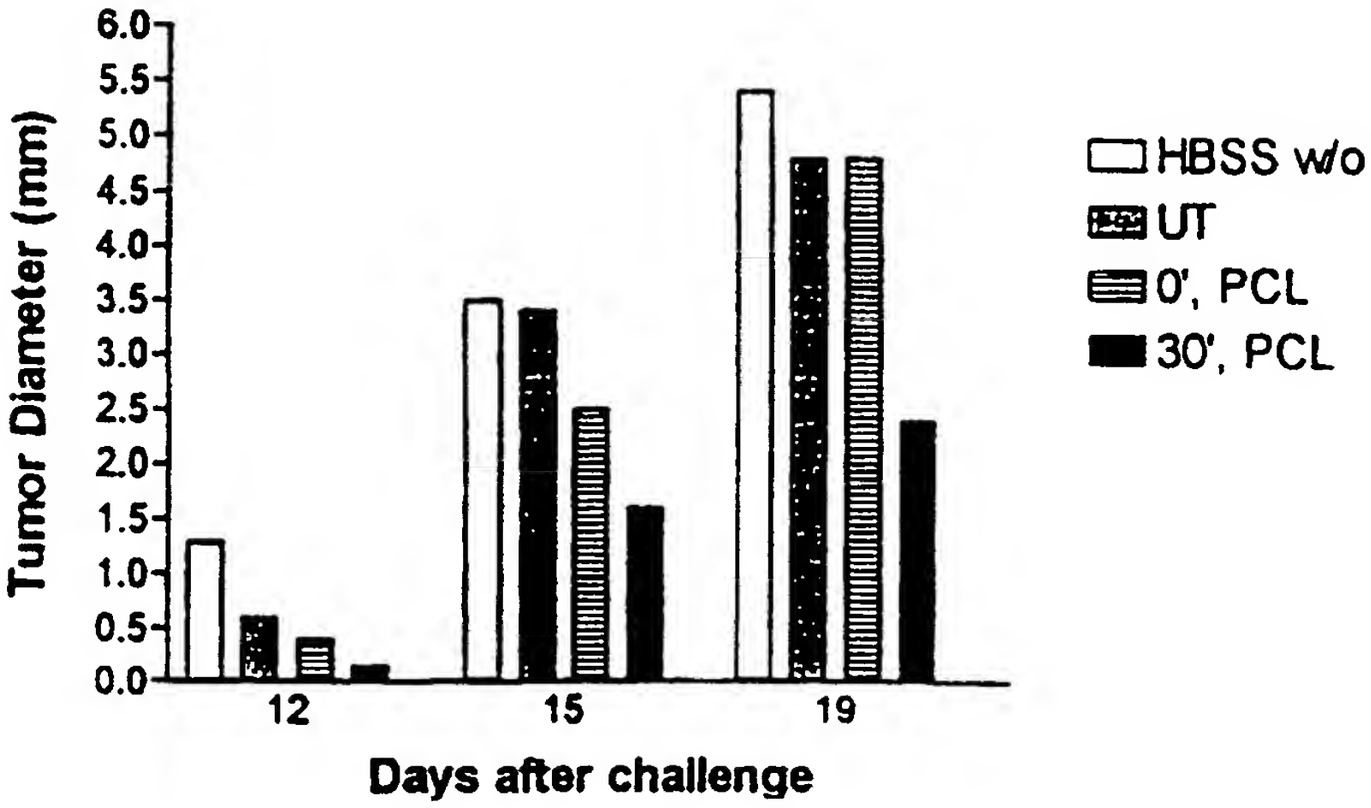

6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Tumor Diameter (mm)
12
15
19
Days after challenge
HBSS w/o
UT
0', PCL
30', PCL

FIG. 6

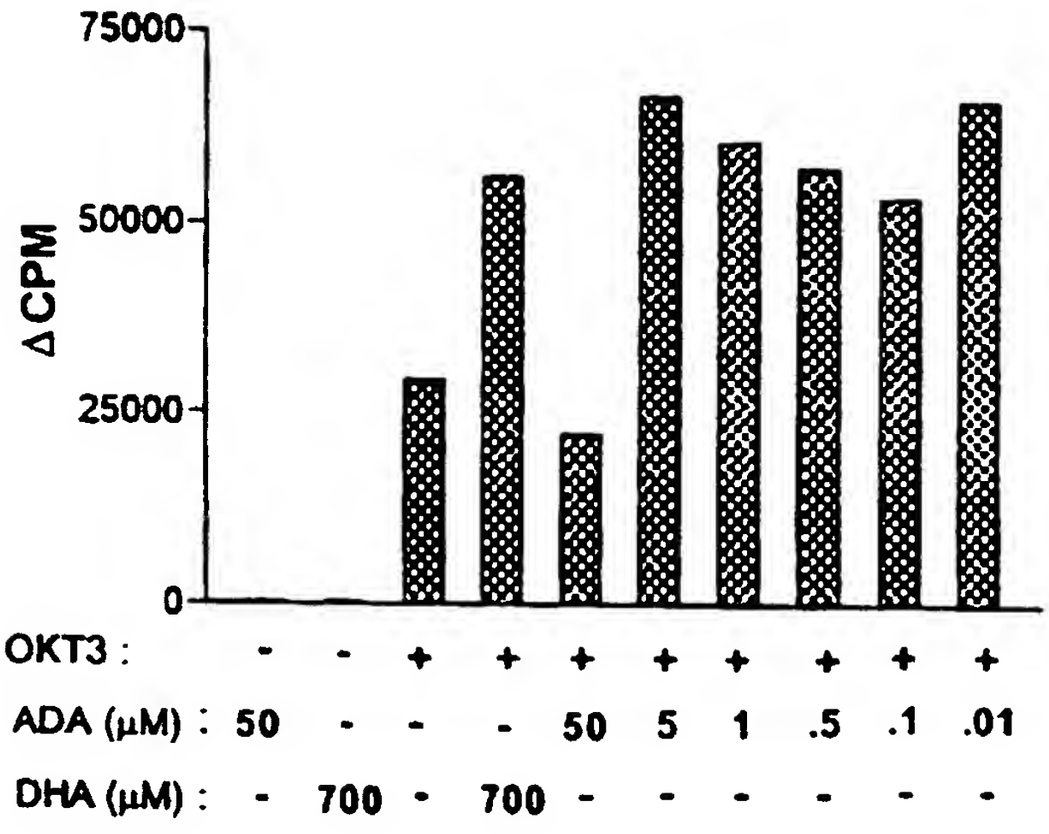
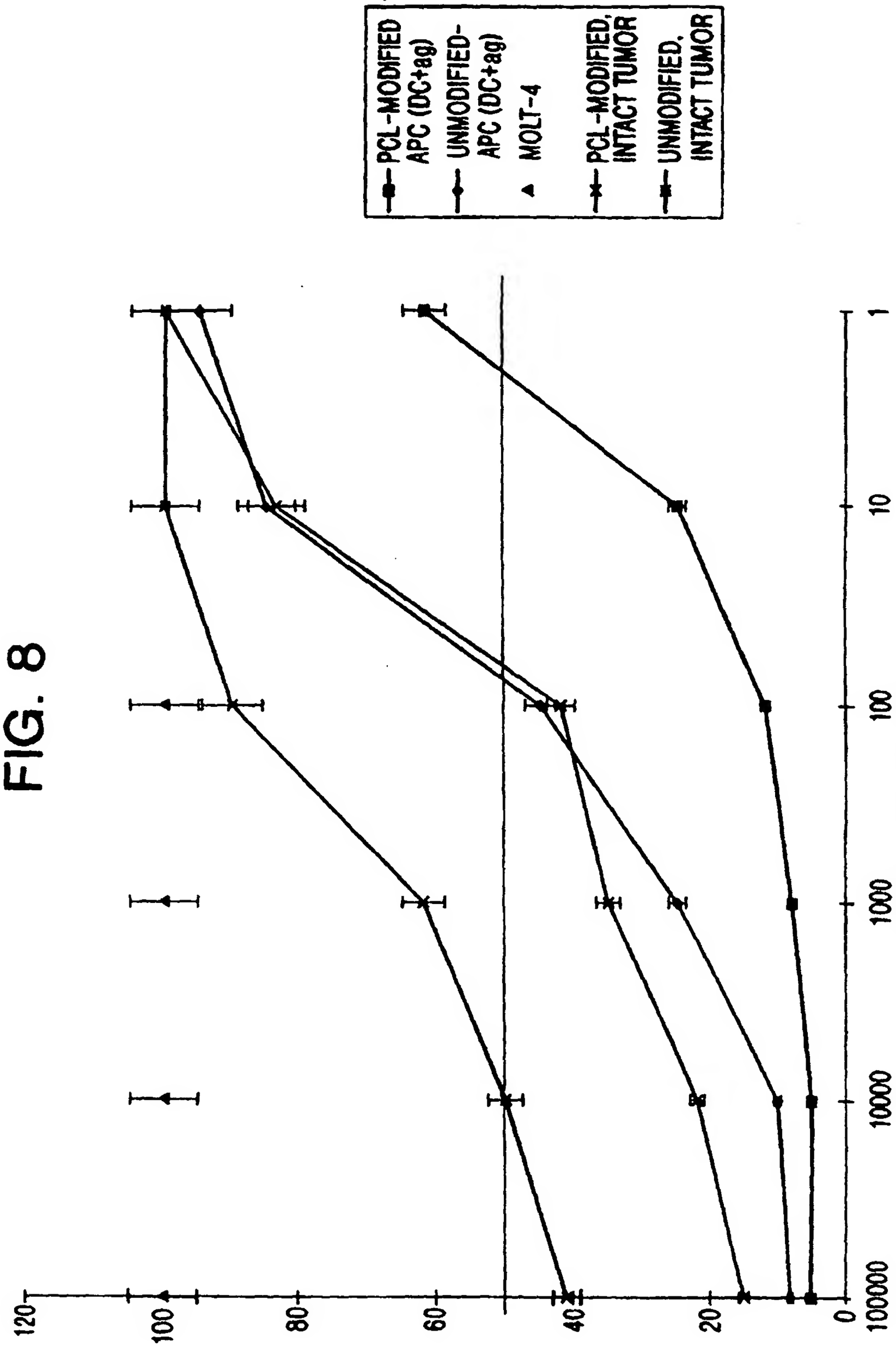

75000
50000
25000
0
ΔCPM
OKT3 : - - + + + + + + + +
ADA (μM) : 50 - - - 50 5 1 .5 .1 .01
DHA (μM) : - 700 - 700 - - - - - -

FIG. 7

FIG. 8


PCL-MODIFIED APC (DC+ag)
UNMODIFIED-APC (DC+ag)
MOLT-4
PCL-MODIFIED, INTACT TUMOR
UNMODIFIED, INTACT TUMOR
% OF KILLED TUMOR CELLS (AUTOLOGOUS B CELL LYMPHOMA)
120
100
80
60
40
20
0
1000
100
10
OF CELLS SEEDED/WELL
10000
100000

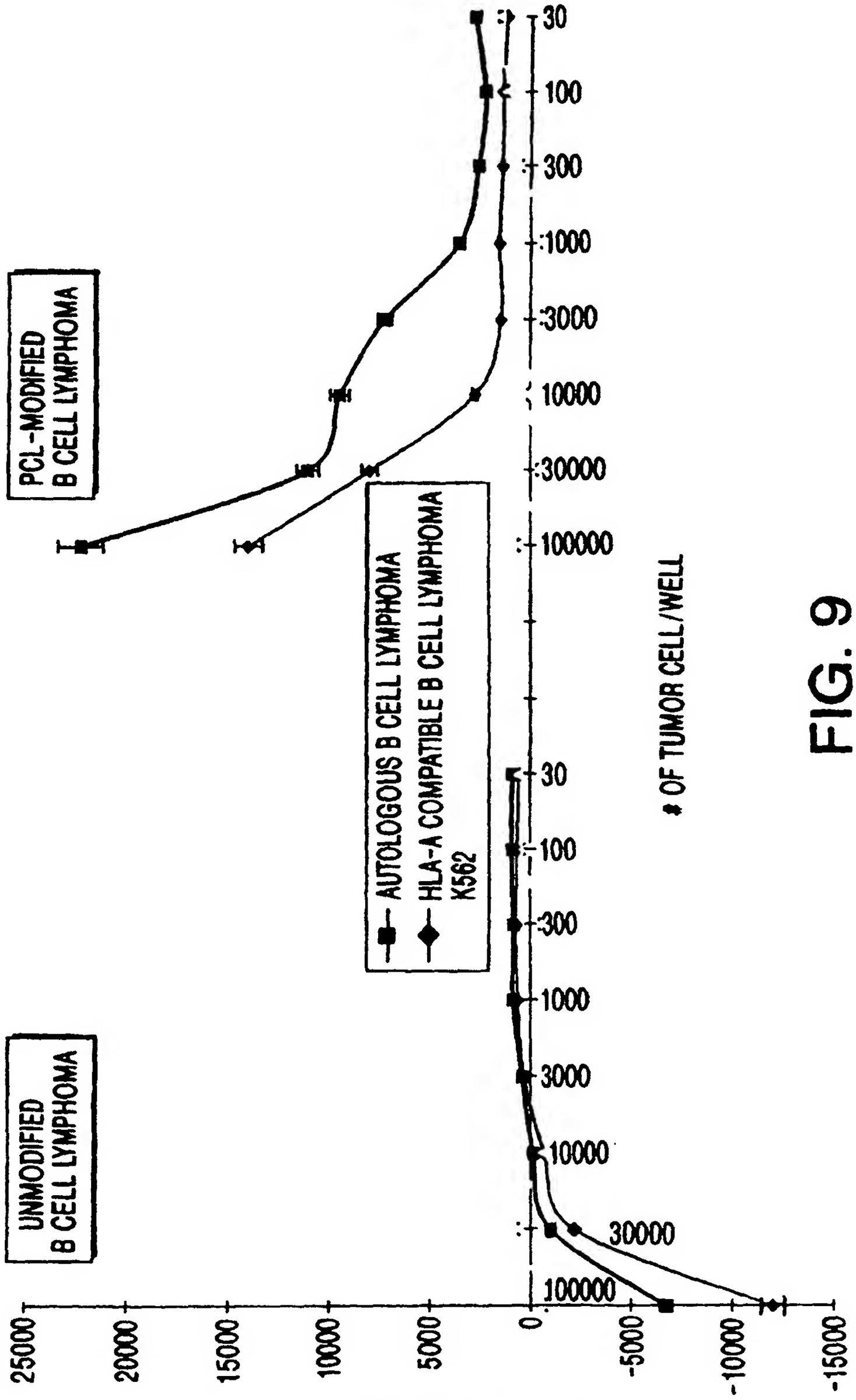

PCL-MODIFIED B CELL LYMPHOMA
AUTOLOGOUS B CELL LYMPHOMA
HLA-A COMPATIBLE B CELL LYMPHOMA
K562
UNMODIFIED B CELL LYMPHOMA
30
100
300
1000
3000
10000
30000
100000
25000
20000
15000
10000
5000
0
-5000
-10000
-15000
3H-THYMIDINE INCORPORATION (cpm)
OF TUMOR CELL/WELL

FIG. 9

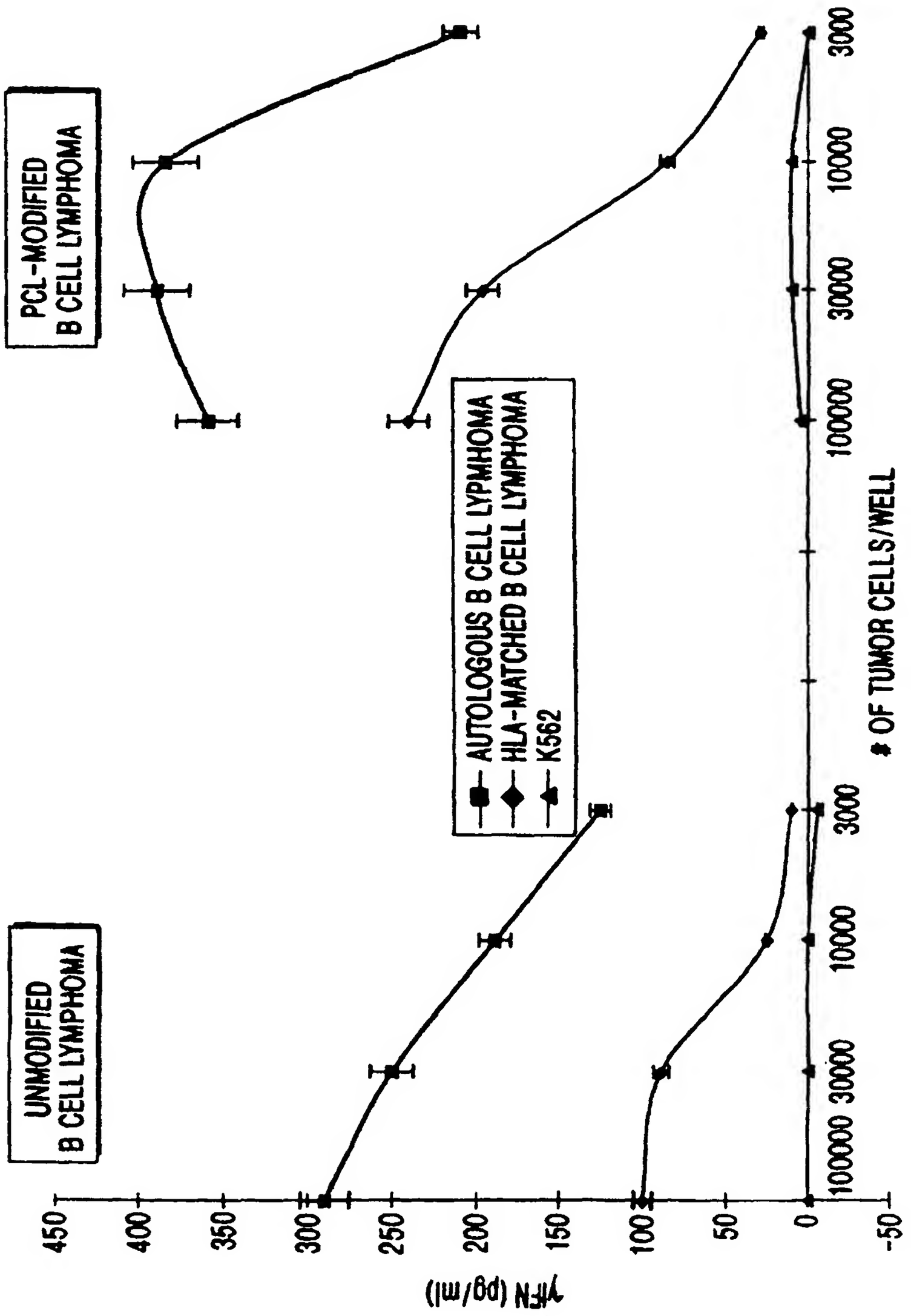

PCL-MODIFIED B CELL LYMPHOMA
UNMODIFIED B CELL LYMPHOMA
AUTOLOGOUS B CELL LYPMHOMA
HLA-MATCHED B CELL LYMPHOMA
K562
OF TUMOR CELLS/WELL
3000
10000
30000
100000
γIFN (pg/ml)
450
400
350
300
250
200
150
100
50
0
-50

FIG. 10

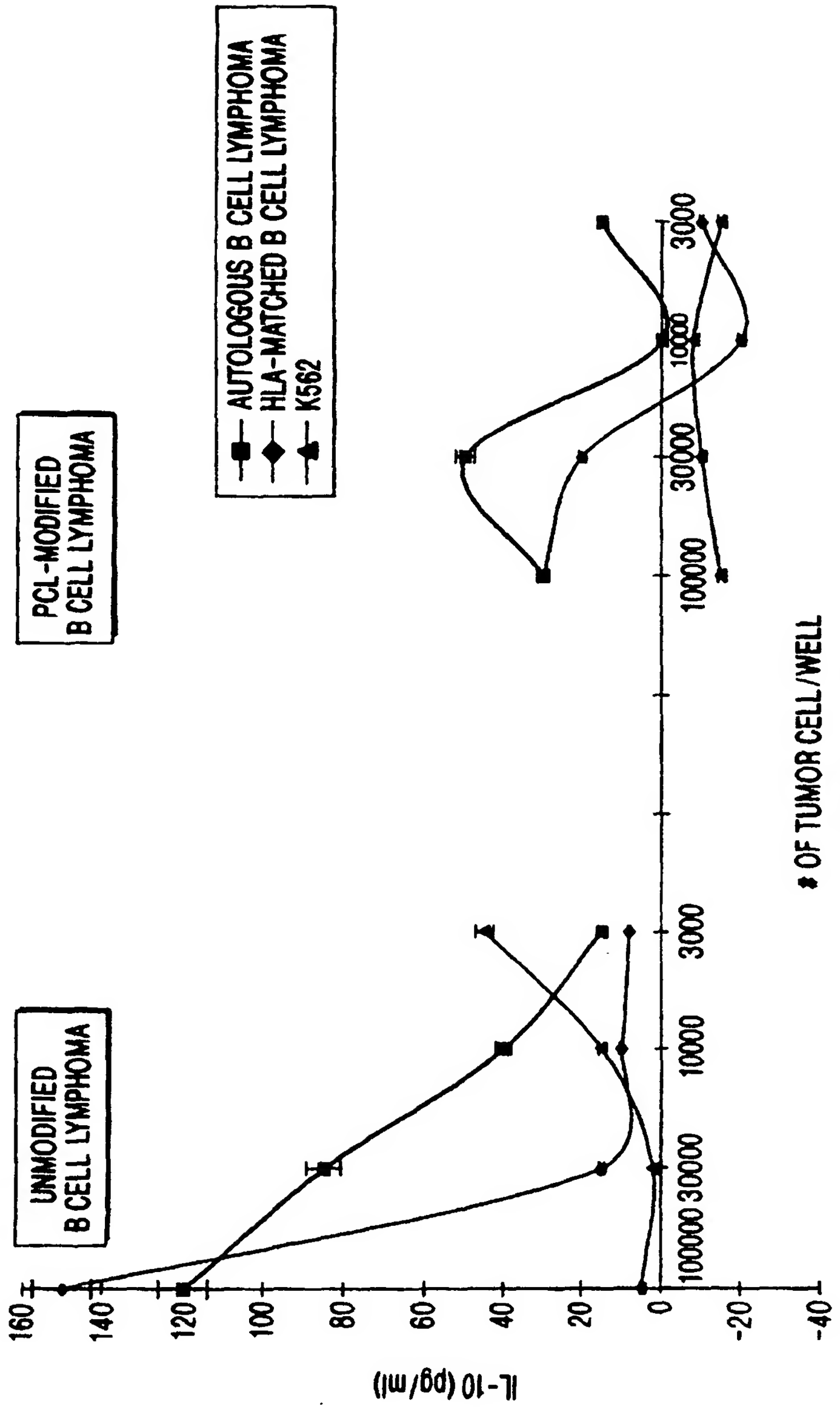

AUTOLOGOUS B CELL LYMPHOMA
HLA-MATCHED B CELL LYMPHOMA
K562
PCL-MODIFIED B CELL LYMPHOMA
UNMODIFIED B CELL LYMPHOMA
IL-10 (pg/ml)
OF TUMOR CELL/WELL
160
140
120
100
80
60
40
20
0
-20
-40
3000
10000
30000
100000

FIG. 11

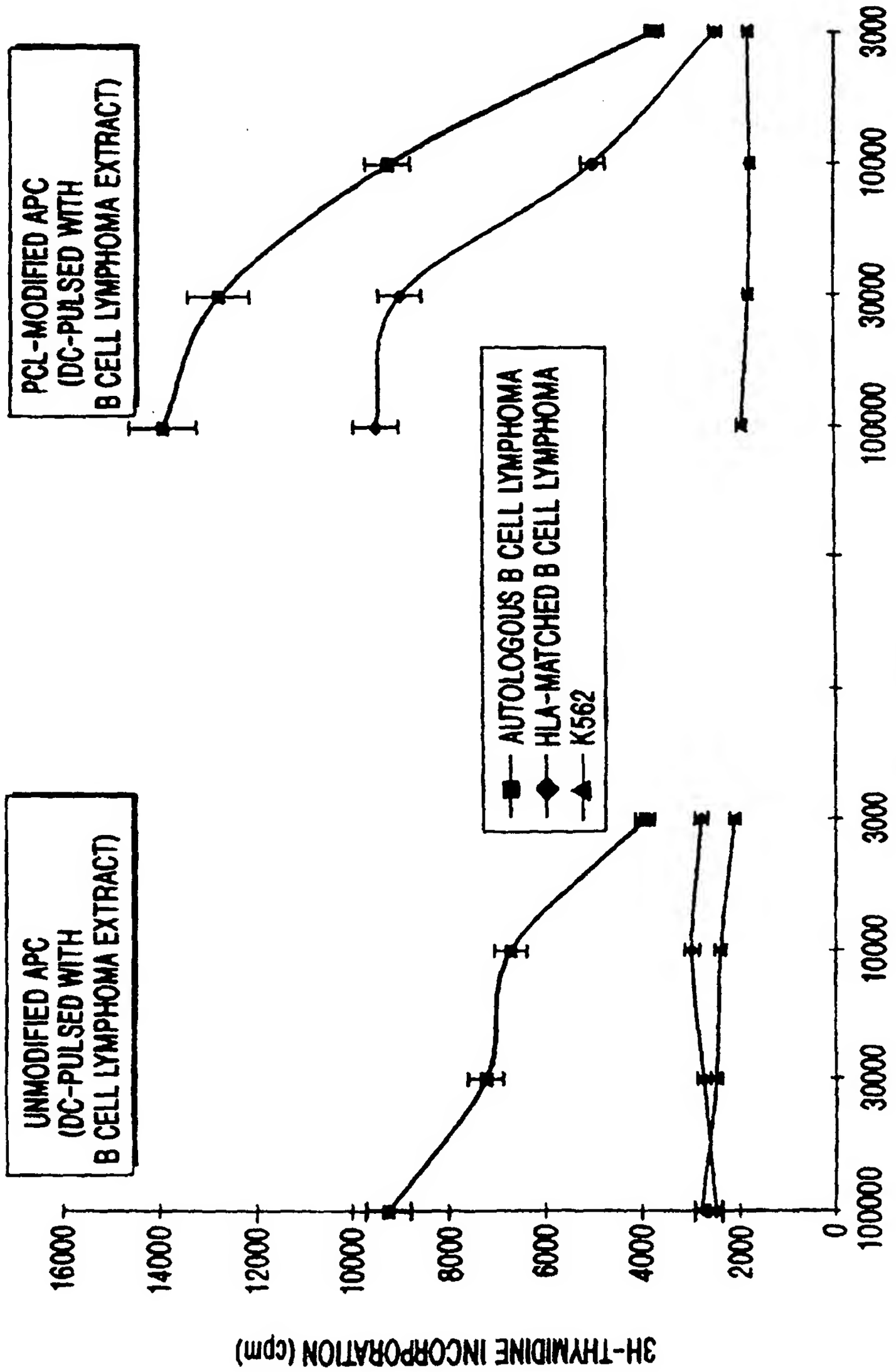

PCL-MODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
UNMODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
AUTOLOGOUS B CELL LYMPHOMA
HLA-MATCHED B CELL LYMPHOMA
K562
3H-THYMIDINE INCORPORATION (cpm)
OF TUMOR CELLS/WELL
16000
14000
12000
10000
8000
6000
4000
2000
0
100000
30000
10000
3000
1000

FIG. 12

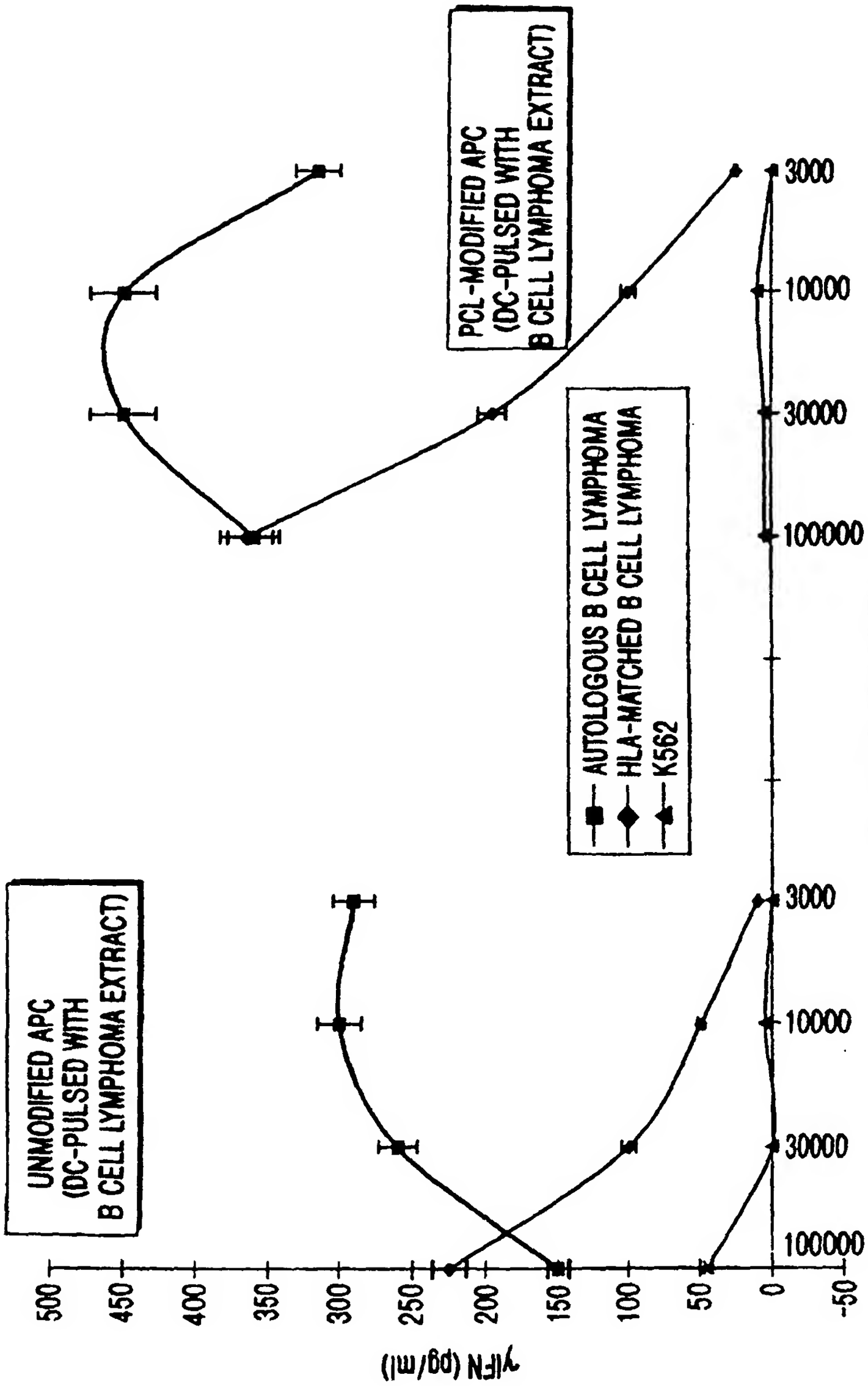

PCL-MODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
AUTOLOGOUS B CELL LYMPHOMA
HLA-MATCHED B CELL LYMPHOMA
K562
3000
10000
30000
100000
OF TUMOR CELLS/WELL
UNMODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
500
450
400
350
300
250
200
150
100
50
0
-50
γIFN (pg/ml)

FIG. 13

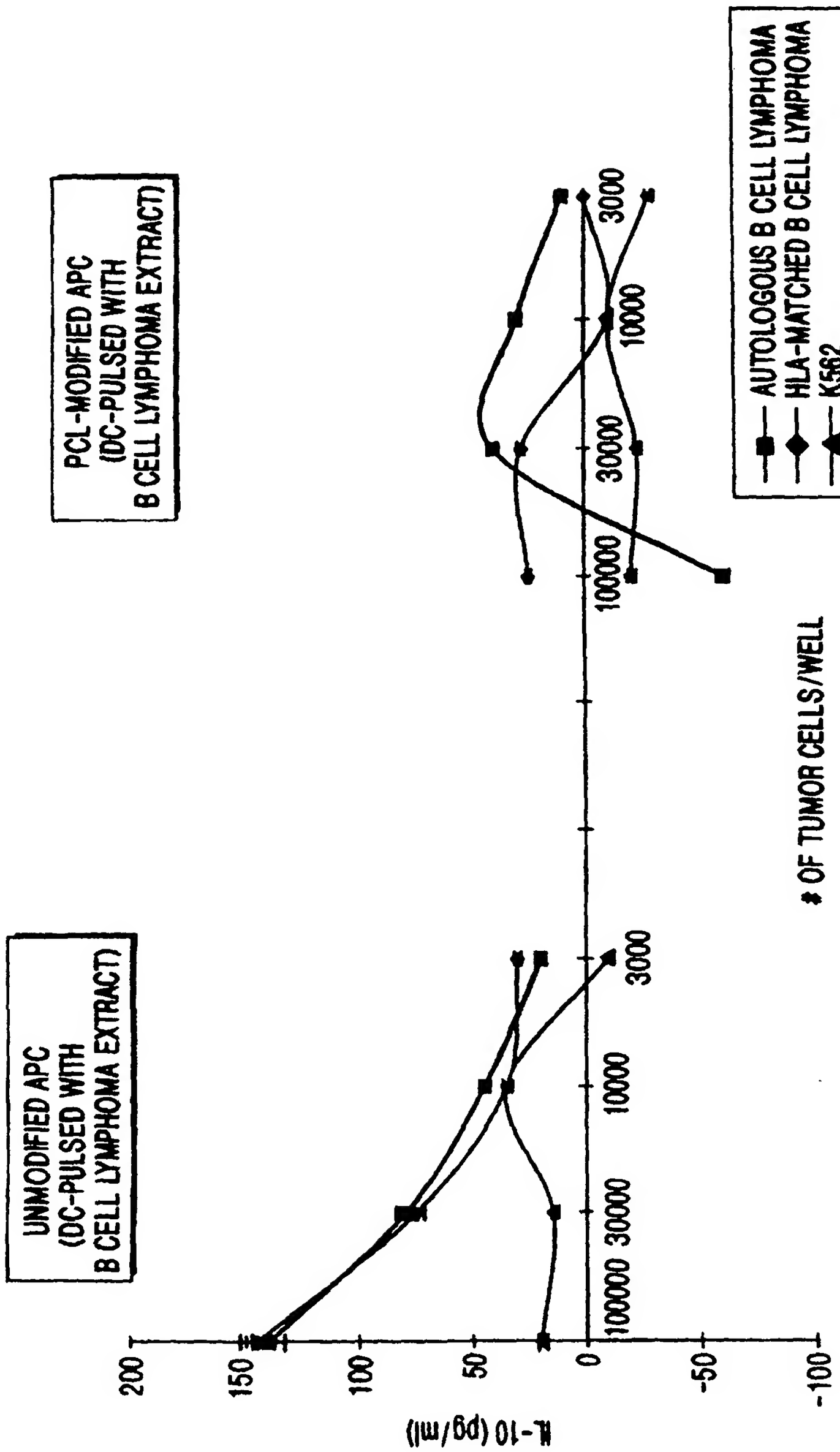

PCL-MODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
UNMODIFIED APC
(DC-PULSED WITH
B CELL LYMPHOMA EXTRACT)
AUTOLOGOUS B CELL LYMPHOMA
HLA-MATCHED B CELL LYMPHOMA
K562
3000
10000
30000
100000
OF TUMOR CELLS/WELL
200
150
100
50
0
-50
-100
IL-10 (pg/ml)

FIG. 14

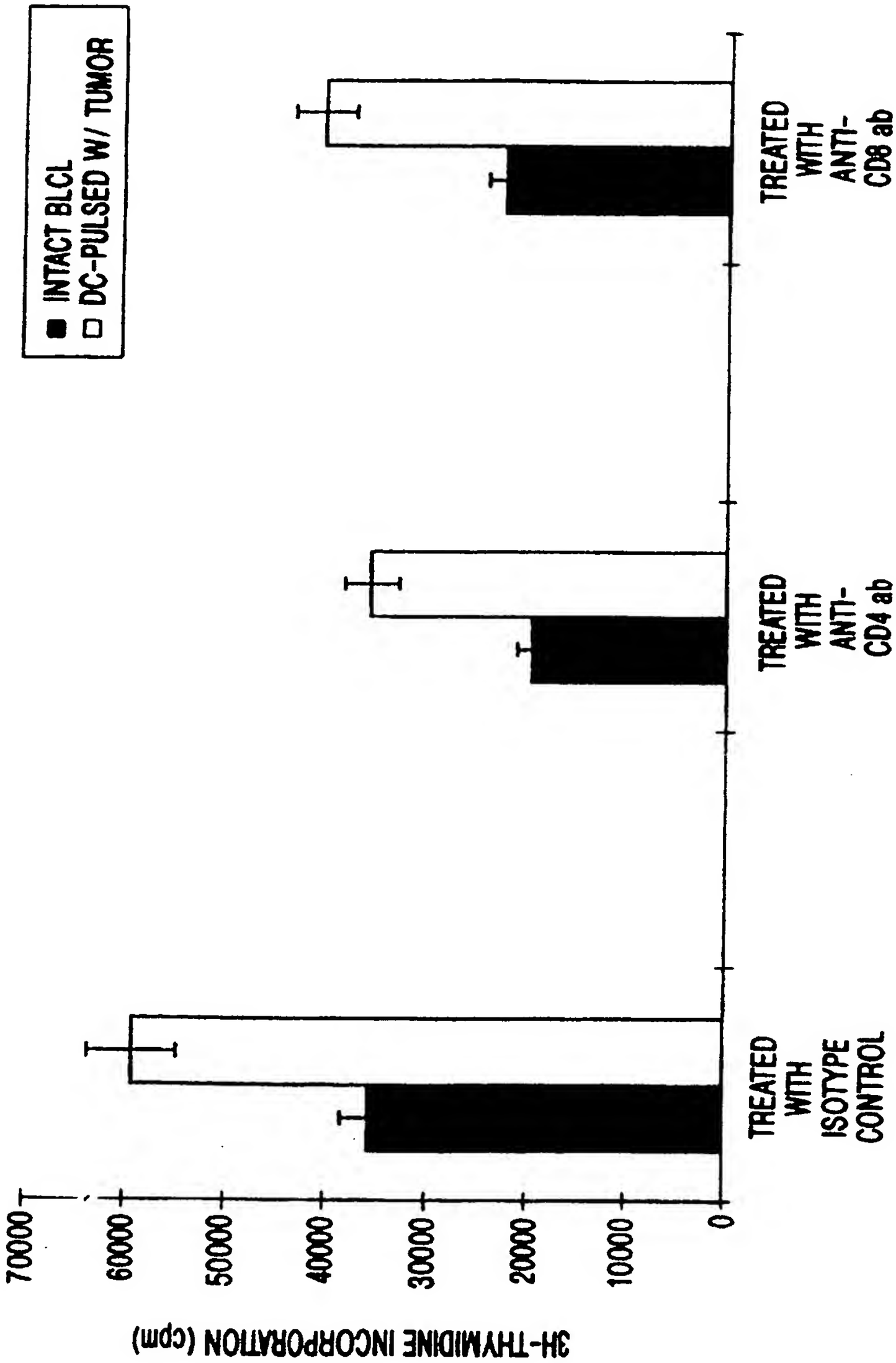

■ INTACT BLCL
□ DC-PULSED W/ TUMOR
TREATED WITH ANTI-CD8 ab
TREATED WITH ANTI-CD4 ab
TREATED WITH ISOTYPE CONTROL
0
10000
20000
30000
40000
50000
60000
70000
3H-THYMIDINE INCORPORATION (cpm)

FIG. 15

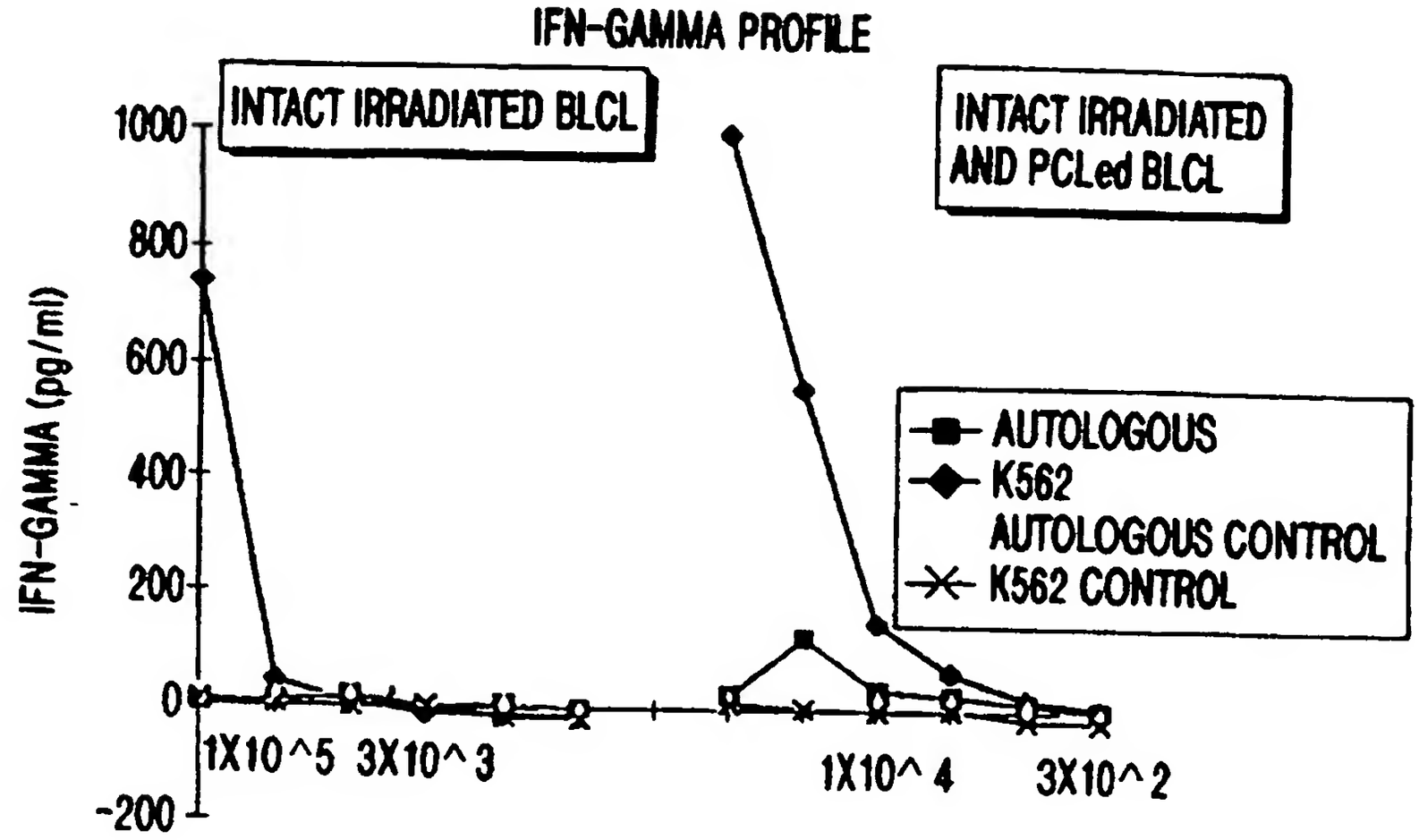


FIG. 16A

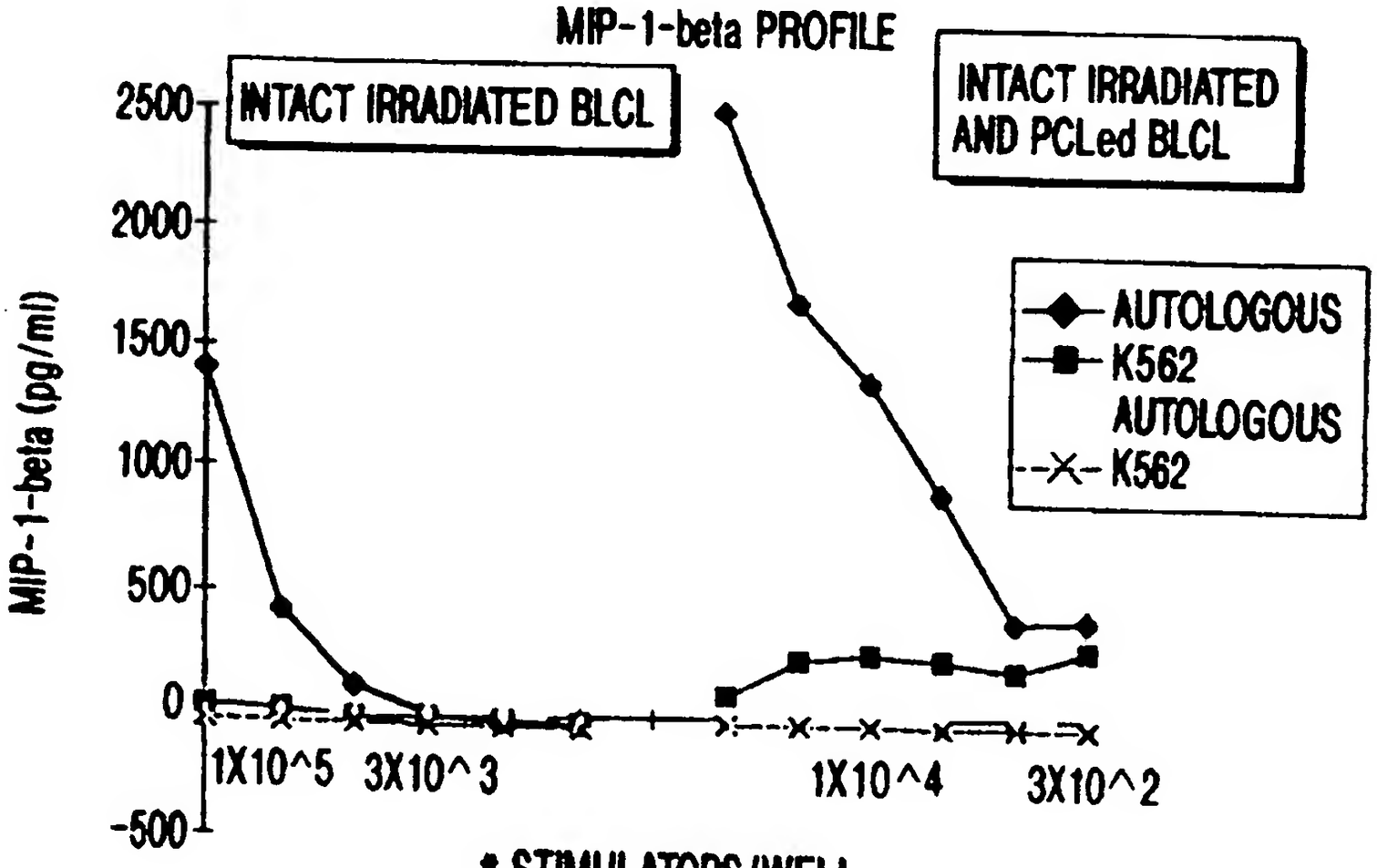


FIG. 16B

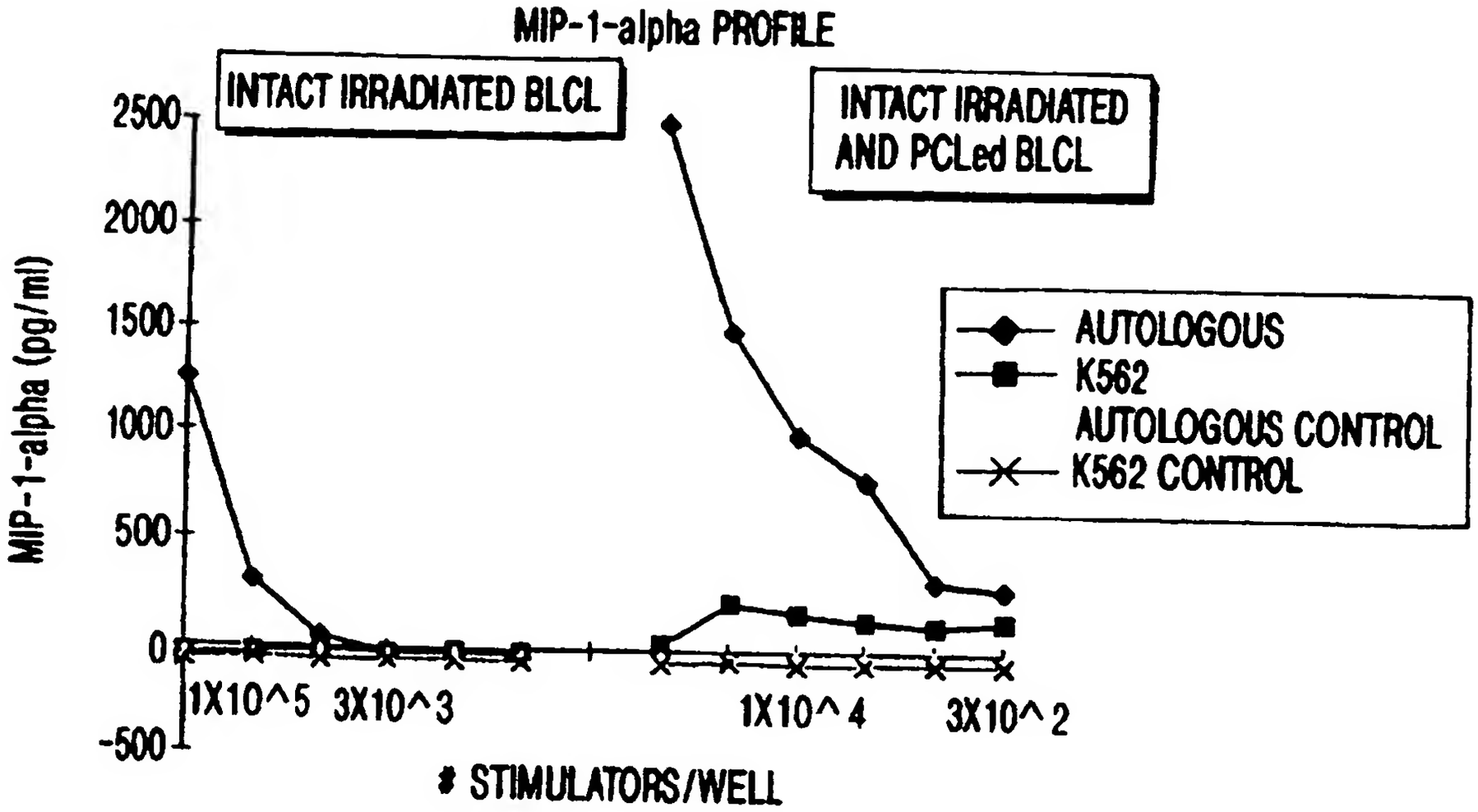


FIG. 16C

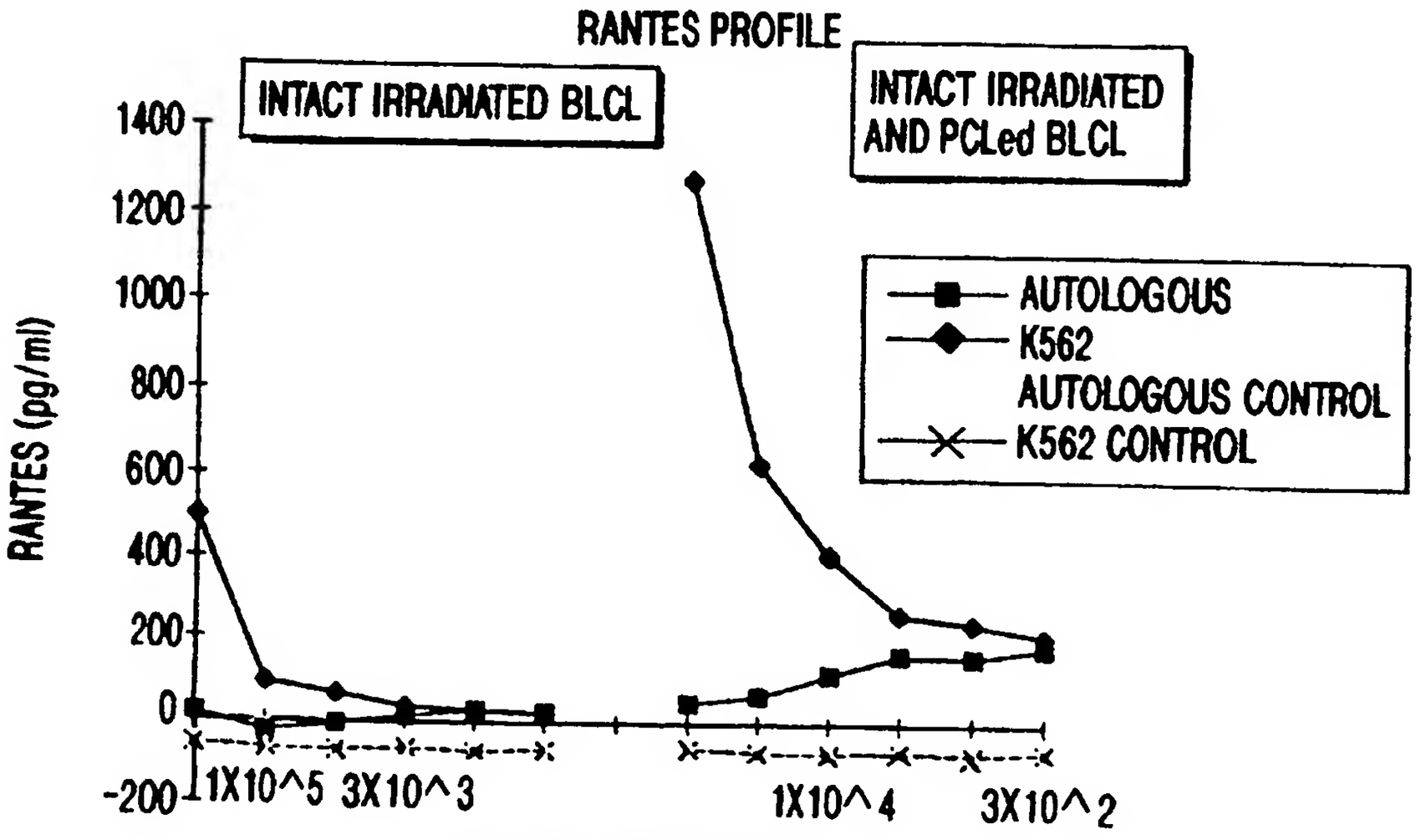


FIG. 16D

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 97/03916

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K39/39 · A61K39/02 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 25664 A (IMMUNOTHERAPY, INC.; YEDA RESEARCH AND DEVELOPMENT CORP. LTD.) 22 August 1996 cited in the application see the whole document	1,2,5-7, 11, 13-17, 19-22, 39-54,81

X	EP 0 615 758 A (YEDA RESEARCH AND DEVELOPMENT CO. LTD.) 21 September 1994 cited in the application	1,2,5-7, 39,41, 42,47-50
Y	see the whole document	3,4, 8-38,40, 43-46, 51-81

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 June 1997	17.07.97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	**Authorized officer** Olsen, L

Form PCT ISA 210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/03916

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CANCER IMMUNOLOGY IMMUNOTHERAPY, vol. 36, no. 5, 1 January 1993, pages 293-299, XP000576722 RAMAKRISHNA V ET AL: "INCREASED PROJECTION OF MHC AND TUMOR ANTIGENS IN MURINE B16-BL6 MELANOMA INDUCED BY HYDROSTATIC PRESSURE AND CHEMICAL CROSSLINKING" cited in the application	1,2,5-7, 39,41, 42,47-50
Y	see the whole document	3,4, 8-38,40, 43-46, 51-81

X	CANCER IMMUNOLOGY IMMUNOTHERAPY, vol. 36, no. 5, 1 January 1993, pages 300-306, XP000576721 EISENTHAL A ET AL: "INDUCTION OF CELL-MEDIATED IMMUNITY AGAINST B16-BL6 MELANOMA IN MICE VACCINATED WITH CELLS MODIFIED BY HYDROSTATIC PRESSURE AND CHEMICAL CROSSLINKING" cited in the application	1,2,5-7, 39,41, 42, 47-50,81
Y	see the whole document	3,4, 8-38,40, 43-46, 51-80

X	CANCER IMMUNOLOGY IMMUNOTHERAPY, vol. 33, no. 1, 1991, pages 1-8, XP000652266 RAMAKRISHNA V AND SHINITZKY M: "POTENTIATION OF DELAYED-TYPE HYPERSENSITIVITY RESPONSE TO SYNGENEIC TUMORS IN MICE PREVACCINATED WITH CELLS MODIFIED BY HYDROSTATIC PRESSURE AND CROSSLINKING" cited in the application	1,2,5-7, 39,41, 42, 47-50,81
Y	see the whole document	3,4, 8-38,40, 43-46, 51-80

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/03916

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9625664 A	22-08-96	AU 4977896 A	04-09-96
EP 0615758 A	21-09-94	JP 6287147 A	11-10-94
		US 5582831 A	10-12-96